ANALYTICAL BIOCHEMISTRY 4, 269–283 (1962)

Analytical Techniques for Cell Fractions. II. A Spectrophotometric Column Monitoring System

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Received November 9, 1961

INTRODUCTION

To follow changes in the composition of cells and tissues occurring as responses to experimental variables, it is necessary to have available rapid automated analytical systems for the major cell constituents. Initially it is prudent to develop those systems that have the widest application. Continuous recording of absorbance of column effluents at one or several wavelengths in the ultraviolet range allows the quantitation not only of proteins, nucleotides, and nucleotide derivatives (4), but also of peptides (with or without aromatic groups) (1–3, 5), and, as will be shown in a subsequent paper, lower fatty acids. In addition, enzymic activities producing changes in ultraviolet-absorbing substrates or cofactors may be monitored.

In this paper the details of two ultraviolet absorbance recording systems are reported. Subsequent papers will deal with methods using them for analyzing specific mixtures.

ANALYTICAL SYSTEMS

Of the four ultraviolet-monitoring systems that we have investigated, two have been found useful for routine analysis.

Single-Beam, Two-Wavelength Systems

The system we have used for more than 2 years (Fig. 1) includes either two Beckman DU spectrophotometers, with spectral energy adapters[2] and power supplies,[3] or one DU and one DUR spectrophotometer.[4] While the latter is not useful below 216 mμ, its electronic stability

[1] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

[2] Spectral energy adapter, Catalogue No. 5800, Beckman Instruments, Inc., Fullerton, Calif.

[3] Power supply, Beckman Catalogue No. 23700.

[4] Available only on special order from Beckman.

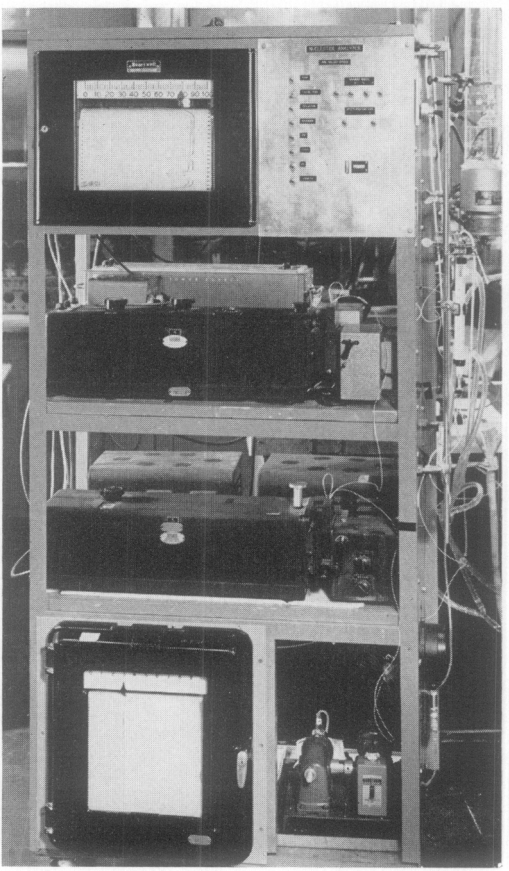

FIG. 1. Single-beam, two-wavelength system. Beckman DU and DUR spectrophotometers (center) are arranged to record absorbance at two wavelengths on the multipoint recorder (top). A recording pH meter with a small-volume electrode assembly is shown in the lower left, while the pump is mounted in the lower right. Columns and gradient-producing devices are mounted on the bars along the right side of the instrument. Control panel (upper right) allows control of each component separately.

is of considerable advantage. The spectrophotometers were attached to a 10-mv Honeywell Multipoint Recorder[5] having a chart speed of 2 in./hr, a 2.5-sec balancing motor, and a 5-sec printing time. The voltages to the spectrophotometers and the recorder were stabilized by a constant-

[5] Model Y 153X67-V12H-II-III-(H16)-A8A9K 12-point cyclic balance strip-chart recorder with a dual nonshorting 12-point selector switch, a separate chart drive motor to obtain a chart speed of 2 in./hr, and a 4× amplifier. Available from Minneapolis Honeywell Inc., Minneapolis, Minn.

voltage transformer.[6] The flow cells (0.2 and 1.0-cm light path)[7] and methods for connecting them to fine plastic tubing have been described (2).

By careful tube selection and maintenance it is possible to obtain very stable baselines. Since considerable drift is seen when the instruments are first turned on, it is our practice not to turn them off unless they are to be idle for more than three days.

The great advantage of these simple single-beam systems is that any two wavelengths from 205 mμ to the near-infrared may be monitored. However, for very prolonged runs (approximately 25 hr) they show a small drift in the dark-current (infinite absorbancy) settings. For this reason a new and relatively inexpensive double-beam spectrophotometer has been modified for flow monitoring.

Double-Beam Monitoring System

The completed system is shown in Fig. 2. A Beckman DB spectrophotometer was modified by the addition of the pneumatic wavelength-shifting mechanism shown in Figs. 3 and 4. A piston, attached through a linkage to the wavelength dial, moves the dial alternately from 260 to 280 mμ (other wavelengths 20 mμ apart may also be selected) in synchrony with the printing cycle of the recorder (Fig. 5). An additional 12-point nonshorting switch[8] is mounted in the recorder and is driven by the same mechanism moving the 12-point circuit selector switch. The second switch actuates alternately two power relays[9] that energize the solenoid valves,[10] which, in turn, drive the pneumatic wavelength-shifting mechanism. The baselines for two wavelengths may be independently adjusted using the arrangement schematically presented in Fig. 5.

If absorbancy at only two wavelengths is to be recorded, the second 12-point switch may be omitted and the signal fed directly into the recorder, in which instance the baseline adjusting circuit cannot be used. The 12-point switch normally in the recorder may then be used for

[6] Harmonic-neutralized, constant-voltage transformer Model CVH-1, Sola Electric Co., Chicago 50, Ill.

[7] Available as Oak Ridge flow cells from Pyrocell Mfg. Co., 207–11 E. 84th St., New York 28, N. Y., or Quaracell Products Co., 401 Broadway, New York 13, N. Y.

[8] Additional 12-point thermocouple switch available with mounting bracket from Minneapolis Honeywell, Inc.

[9] Relay, 115-volt, 60-cycle, 20-amp contact SPDT, Ward Leonard Electric Co., 45 South Street, Mount Vernon, N. Y., No. 105-6512.

[10] Solenoid valve No. V5D18360-V-10, 115-volt, 60-cycle, ⅛-inch NPT, 3-way, bleed-type, Skinner Electric Valve Division of Skinner Chuck Co., 100 Edgewood Ave., New Britain, Conn.

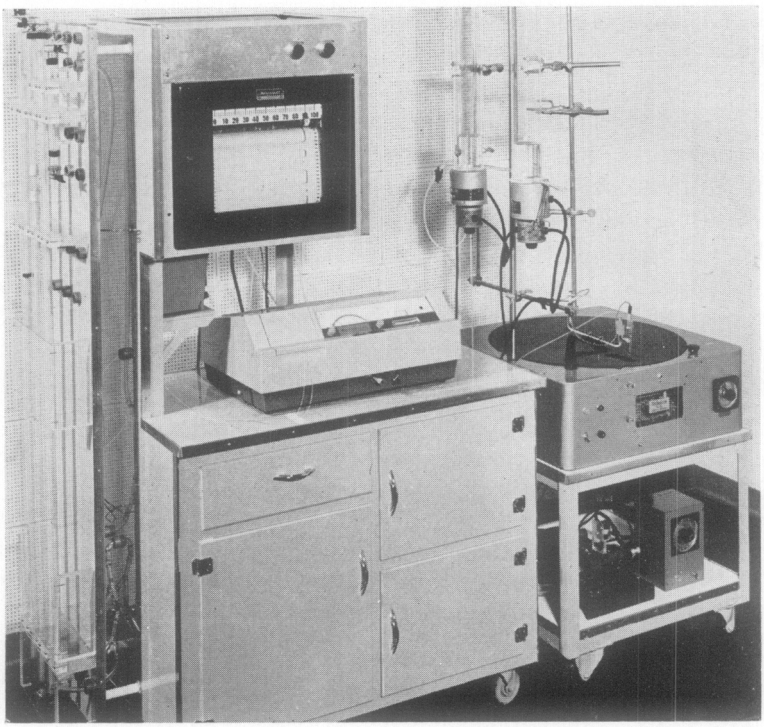

FIG. 2. Complete analytical system for nucleotides, proteins, or other ultraviolet-absorbing materials. Circulating water bath is mounted inside the cabinet in lower left compartment; pump (if not mounted on fraction collector) is in lower right space; hydrogen lamp power supply is mounted in upper right section. Baseline adjusting potentiometers are mounted above the multipoint recorder. The controlling multimeter is mounted between the recorder and the spectrophotometer table. Ion-exchange columns are mounted on a panel projecting from the main cabinet (left) a sufficient distance to allow the column jacket water lines to be easily reached.

wavelength shifting. However, in the present instrument, provision is made for recording pH, conductivity, and radioactivity, in addition to absorbancy, on the same chart. These signals must also pass through a selector switch.

We have used Milton Roy chromatographic pumps.[11] Connections to the pump are made with 3/8-in. Tygon tubing through swagelock connections.[12] In early work, salt rings occurred around these connections

[11] Minipump, CHMMI-B-29R, Milton Roy Co., 1300 E. Mermaid Lane, Philadelphia 18, Pa.

[12] Swagelock fitting No. 600-1-2-316, Crawford Fitting Co., 884 E. 140th St., Cleveland 10, Ohio.

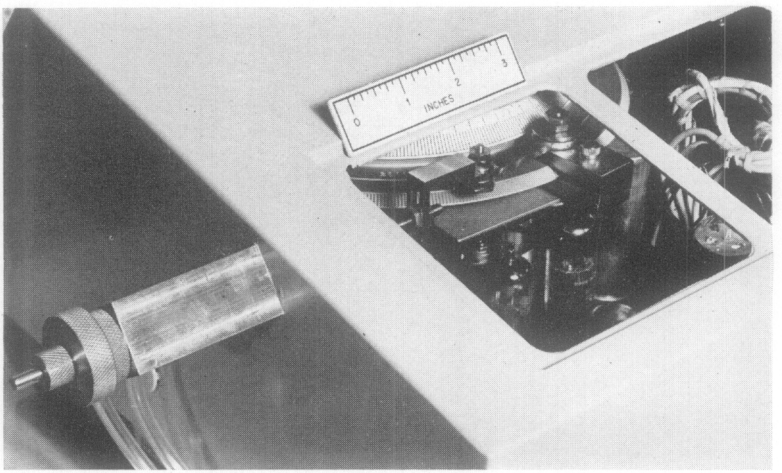

FIG. 3. Air-operated, wavelength-shifting mechanism. The air piston mounted outside the spectrophotometer housing moves a shaft linked to the wavelength dial. Stops are provided in the piston housing to adjust the travel of the piston and to position accurately the wavelengths to be read, in this case 260 and 280 mμ.

because sealing compounds were avoided. However, no leakage is observed when the male threaded parts are wrapped in Teflon tape.[13]

In a specific system, which will be described in a subsequent paper, a change in pumping rate occurs during chromatography. This is accomplished by lifting a flat stainless-steel spacer from between the cross head and set screw with a small, continuous-duty solenoid,[14] as shown in Fig. 6. The solenoid is actuated by a timer that energizes it at the end of a preset time.

Connection from the pump to the top of the chromatographic column is through a polyvinyl chloride ball connector fitted with a silicone rubber "O" ring.[15] All columns are jacketed, with 18/9 top ball connections and 12/2 effluent ball connections. Both glass frit columns[16] and Teflon frit columns[17] of 0.9-cm i.d. were used. Column temperatures were controlled with a circulating constant-temperature bath[18] equipped with

[13] Thread-tape, Crane Packing Co., Morton Grove, Ill.

[14] No. 1R-11-Cont-115-Ac, 60-cycle solenoid, Guardian Electric Mfg. Co., 1621 W. Walnut St., Chicago 12, Ill.

[15] Connector assembly PVC ball 18/1, tubing to column, part No. 120-9100 with "O" ring No. 120-9036, Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif.

[16] Available from Scientific Glass Apparatus Co., Bloomfield, N. J.

[17] Available from Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif.

[18] Constant-temperature circulating bath, Catalogue No. 3050, Labline Inc., 3070-82 W. Grand Ave., Chicago 22, Ill.

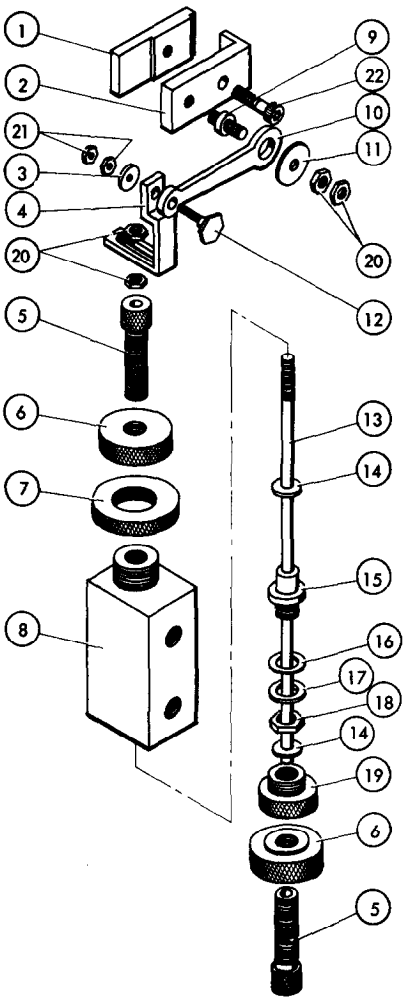

FIG. 4. Assembly of wavelength-shifting mechanism. (1) Lower jaw of wavelength dial clamp. (2) Upper jaw of wavelength dial clamp. (3) Washer. (4) Bracket connecting piston shaft to driving arm. (5) Adjustment for upper wavelength to be read. (6) Lock nut for 5. (7) Nut for holding piston to spectrophotometer housing. (8) Air piston cylinder. (9) Bearing connector between wavelength dial-clamp and driving arm. (10) Driving arm. (11) Washer. (12) Bolt with bearing surface for connecting parts 4 and 10. (13) Air piston shaft. (14) Teflon washers. (15) Piston. (16) Leather washer. (17) Brass washer. (18) Brass piston nut. (19) Piston cylinder end closure. (20, 21) Brass nuts. *Note:* Number corresponds to parts on engineering drawings available from author.

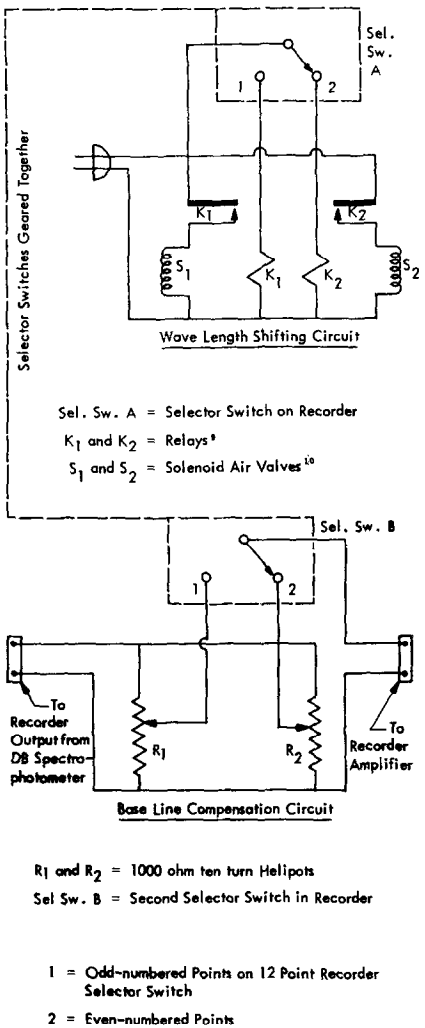

FIG. 5. Circuit for independent adjustment of two baselines and circuit for wavelength switching.

coils for rapid cooling of the bath with tap water. All fluid-handling lines beyond the columns are of Teflon[19] joined as previously described (2), or by heat-shrinking expanded Teflon tubing.[20]

[19] No. PF 22 Teflon tubing, Pennsylvania Fluorocarbon Co., Inc., 1115 N. 38 St., Philadelphia, Pa.

[20] Expanded Teflon AWG# 19 STD Natural tubing available from Pennsylvania Fluorocarbon Co., Inc. When heated, tubing will shrink to form a tight bond with No. PF 22 Teflon tubing.

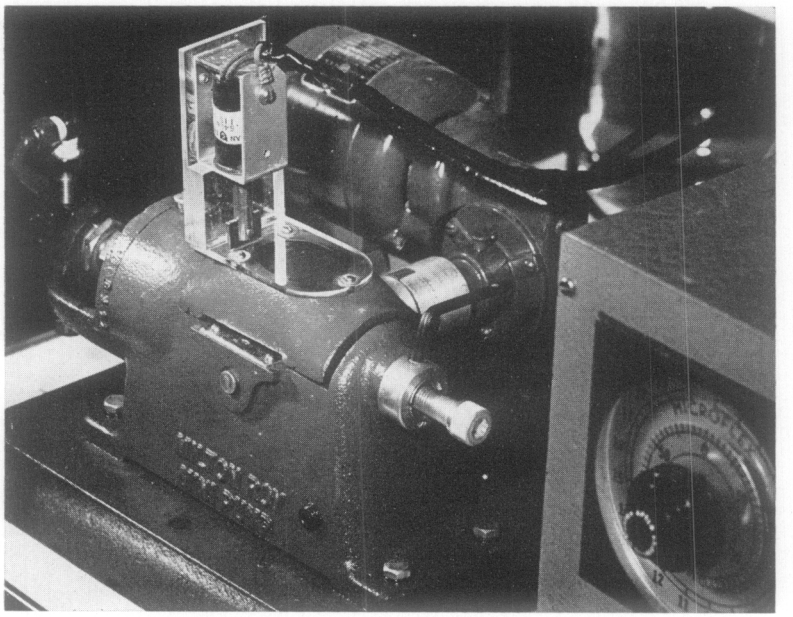

FIG. 6. Timer-operated mechanism for changing pumping rate. At preset time, the timer energizes the solenoid, which raises the stainless-steel plate from between the cross head and set screw, increasing the pump stroke. The blade is shown in down position.

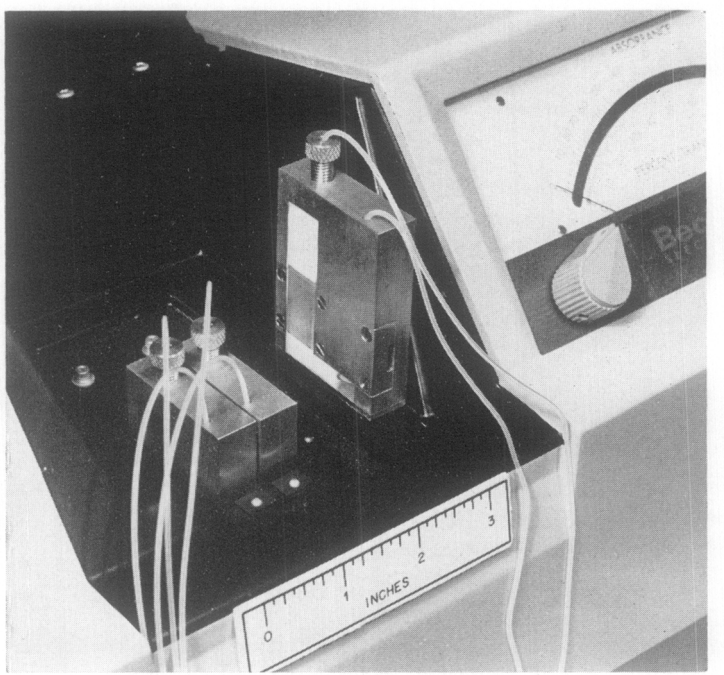

FIG. 7. Quartz flow cell assemblies.

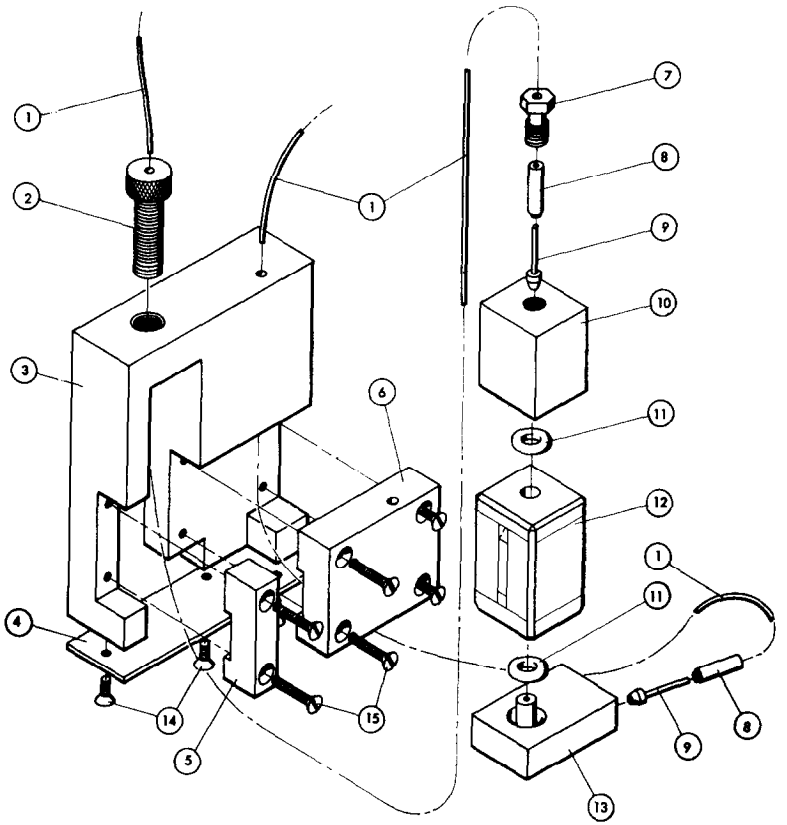

FIG. 8. Assembly of quartz flow cells (see *Note,* Fig. 4). (1) Teflon tubing. (2) Upper pressure plate adjustment screw. (3) Cuvette housing block. (4) Bottom plate of cuvette housing. (5) Front beam guide. (6) Rear beam guide. (7) Swivel fitting pressure screw. (8) Swivel fitting sleeve. (9) Swivel fitting. (10) Upper pressure plate. (11) "O" ring. (12) Quartz cuvette. (13) Lower pressure plate. (14, 15) Screws.

The quartz flow cells (Figs. 7 and 8) are modifications of those previously described (2) with optical paths of 0.2 and 1 cm and volumes of 0.056 and 0.25 ml, respectively.[7] Connections are made through Teflon end blocks and swivel connections.[21] The free area for the light beam is 0.2 by 1 cm. The cell holder completely fills the cell space of the Beckman DB spectrophotometer. For baseline correction the elution fluid may flow through the reference cell before being pumped through the column.

Flow Rate. Quantitative chromatography requires that the flow through

[21] Swivel fitting assembly for standard wall Teflon tubing A.W.G. 22 0.012-in. wall, part 120-311, Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif.

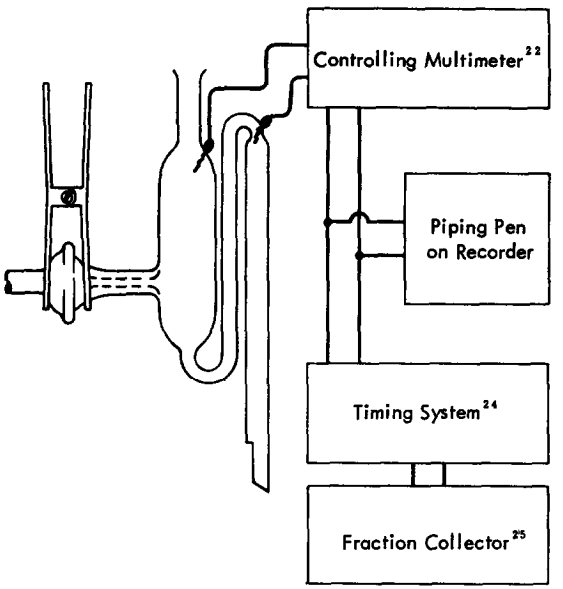

FIG. 9. Flow rate measuring system. Connection at left is to line leading from cuvette. The top is open. The siphon at bottom drains into the fraction collector tube.

the column be precisely known. When a reagent stream is added to the column effluent, as in amino acid analysis, the flow rate both from the column and of the reagent stream must be known, must be constant, and must have a constant ratio. In ultraviolet absorption analysis, however, the only requirement is that the flow rate during the elution of a given peak be known. The flow rate need not be constant from run to run, nor even be constant during the course of a run. Great operational flexibility is therefore obtained by constantly recording the flow rate.

Flow rates may be measured by continuously weighing the effluent on a recording balance or by using a number of commercially available flowmeters. A method based on a calibrated siphon was chosen here, however, because the device is nearly independent of rate and solution density, and because it may be used to actuate a fraction collector. We devised a simple, standard-component method for producing a pulse when the siphon emptied by attaching a controlling multimeter[22] to two platinum wires attached to the siphon,[23] visible above the fraction col-

[22] Controlling Multimeter, Assembly Products, Inc., Chesterland, Ohio, adjusted to measure resistance.

[23] Siphons were obtained from Technicon, Inc., Chauncey, N. Y., and are available in a range of sizes. Platinum wires are fused into the glass in the drain arm and

lector in Fig. 2. Since the actual resistance may be observed constantly, a setting can be obtained that allows actuation with a variety of salt concentrations, but leaves the circuit open when the siphon is not draining.

The multimeter actuates a right-hand piping pen on the recorder, and a collector timer (Fig. 9). The collector timing system is started by a pulse from the controlling multimeter, which, in turn is actuated by the siphon. The timing system runs 20 sec (to allow the siphon to drain fully) and then delivers an impulse to the fraction collector, which has been switched to Count.[26] In this way, constant-volume collections may be made and the flow rate recorded during the entire chromatographic run. The multimeter may also actuate a printing timer, which may record the actual time the siphon drains.

A mounting rack (Fig. 2) holds the constant-temperature bath (lower left), pump (lower right, or under fraction collector), and hydrogen lamp power supply (upper right). Space is provided for four columns, which may be long (150 cm), medium (50 cm), short (15 cm), in any combination. No space or facilities for storing eluting solutions is provided, since requirements differ.

It is a prime rule in the design of automated analytical apparatus that all electrical lines be above and separate from fluid-handling lines. Tubing to the columns exit from the main part of the cabinet a foot or two from floor level and runs up in the readily accessible space between the cabinet and the column mounting board. All lines and connections are within sight and reach. More elaborate flow manifolds for connecting any one of four columns to the spectrophotometer and the rest to drain have been constructed using three precision microvalves.[27] For most applications this is not necessary, however.

Performance of Flow Cells

Since all flow cells contain a finite volume of fluid, it is not possible to avoid completely measurement artifacts when large differences in density, viscosity, or absorbancy occur over a very small volume inter-

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in the body of the siphon so that a current can flow between the two wires only during the period when the siphon is draining.

[24] Series RC timing system, for operating two circuits, for single-cycle, nonrepeating operation with cycle time of 30 sec, Meylan Stopwatch Corp., 264 W. 40 St., New York 18, N. Y.

[25] Technicon Fraction Collector, Technicon, Inc., Chauncey, N. Y.

[26] Detailed circuitry available from the author.

[27] Circle Seal Precision Valve, Model P4 418T, Four Way 3Q61 B obtained from Circle Seal Products Co., Inc., 2181 East Foothill Blvd., Pasadena, Calif.

val. Measurement of the rate of equilibration of the cell contents with the incoming column effluent may be made, but these are of little value in instances in which a small volume of dense fluid (generally the original sample volume containing excess salts and sugars) remains in the cell and convects back through the incoming stream. In the present work the volume of the cells has been reduced as far as is consistent with a good optical cross section. Flow from the bottom of the cell to the top ensures that bubbles are swept out, and results in minimal convective disturbance when gradients of increasing physical density are used for elution.

The correct test with a given system is to observe the performance with the sharpest (contained in the least volume) peaks expected. In our experience these occur at the beginning of the nucleoside-base-nucleotide separation method (4) worked out with the DU-DUR system described

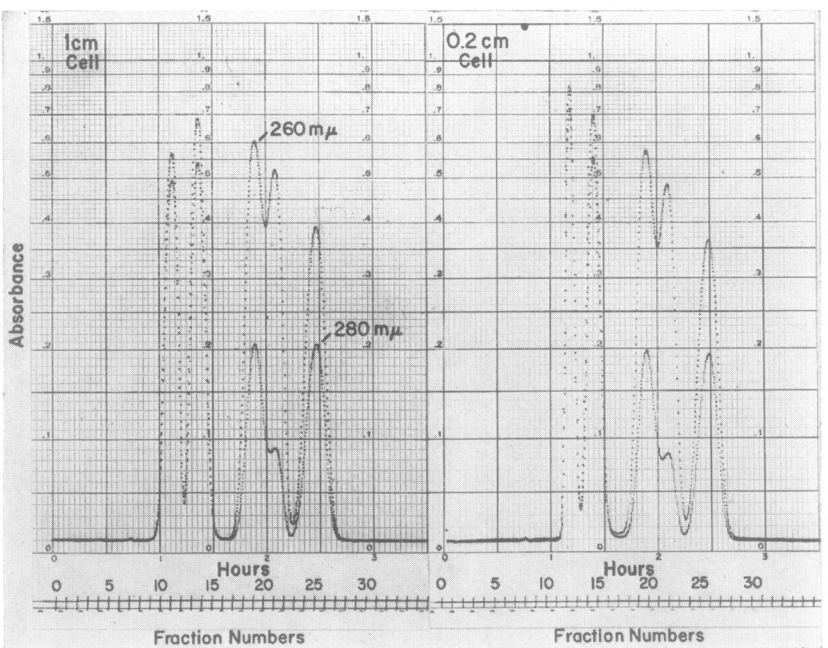

FIG. 10. Comparison of results obtained with 1.0-cm and 0.2-cm light path flow cells. The samples used are described in the text. The elution order is (from left to right) cytosine, cytidine, uridine, uracil, and thymine. These compounds were eluted from a 150 × 0.9 cm Dowex-I X8 column with 0.15 *M* sodium acetate at pH 4.4 at 40°C as described in reference (4). Each mark below the baseline is equivalent to 4.033 ml of column effluent. Absorbance is recorded at 260 and 280 mμ.

here. With a 1-ml sample of a mixture containing 2 μmoles each of cytosine, cytidine, uridine, uracil, and thymine per milliliter with the 0.2-cm cell (0.056-ml cell volume), and 0.2 ml of the same sample with the 1.0-cm (0.25-ml) cell, the recordings shown in Fig. 10 indicate negligible differences between the two cells. The volume width of the first (cytosine) peak is approximately 3 ml. The rest of the peaks are wider and would not be expected to differ. To obtain comparable resolution by reading manually fraction collector samples, a very large number of very small fractions must be collected.

Some difficulty is experienced with the 1-cm cell at the very start of the base-nucleoside-nucleotide analysis when the sample contains a large amount of sucrose. Inverting the cell for about two minutes just after one column volume has passed through eliminates this difficulty. Alternatively, the 0.20- and 1.0-cm cells may be connected in series and the 0.20-cm cell placed in the spectrophotometer until the column volume plus 4–5 ml have passed, during which time the 1.0-cm cell is kept inverted outside the spectrophotometer. The 1.0-cm cell may then be reinserted. If the 0.20-cm cell is to be used for the entire run, no difficulty is experienced. With this single exception no difficulties attributable to the flow cells has been experienced in several hundred runs. The fluid volume below the sintered glass plate in most ion-exchange columns is larger than the volume of the flow cells used here, and is probably a greater source of error than is anomalous flow or back-mixing in the flow cells.

Quantitation of Results

If all of the column effluent containing one ultraviolet-absorbing compound is collected in a single vessel, the amount of that substance may be obtained from the familiar relation

$$\text{mg solute} = \text{MW} \cdot As \cdot \text{ml}/\epsilon \tag{1}$$

where As is the observed absorbance at a given wavelength in a cell of 1-cm light path, and ϵ is the Molar Absorptivity at the same wavelength.

When the data are obtained in the form of recorded Gaussian curves, the following relations may be used to calculate the amount of a given substance present. In the normal density function the relation between the true area under the curve and the area of a triangle obtained by multiplying the height of the curve by the width at half-height is given by:

$$C = G/H \cdot W \tag{2}$$

where G is the true area under the curve, and H and W the height and

width at half height, respectively. Since the area $G = 1$ in the normal density function, and the height is $(2\pi)^{-1/2}$, the width at half-height is given by setting $\Phi(X)$ equal to $(2\pi)^{-1/2}/2$ in the equation:

$$\Phi(X) = \frac{1}{(2\pi)^{1/2}} \exp\{-\tfrac{1}{2}X^2\} \tag{3}$$

which gives $X = 1.177$, and $W = 2.354$. By substitution in Eq. (2), C is found to be 1.064.

In the graphic results, however, the height of the curve is given in absorbancy units (A) and the width at half-height in minutes. The curve may be considered comparable to a volume of fluid V having an absorbance A_V since:

$$A_V \cdot V = H_A \cdot W_{\min} \cdot C \cdot \text{ml/min} \tag{4}$$

and:
$$\text{mg solute} = (\text{MW}/\epsilon) \cdot H_A \cdot W_{\min} \cdot \text{ml/min} \cdot C \tag{5}$$

or:
$$\text{mmoles} = (H_A \cdot \text{W}_{\min}/\epsilon) \cdot \text{ml/min} \cdot C \tag{6}$$

DISCUSSION

While the system described here was intended primarily for work with nucleic acid-derivatives, it is applicable to analysis of a wide variety of other absorbing substances including peptides by far-ultraviolet absorption, or substances absorbing in the visible range. Its usefulness in spectrophotometric methods for enzyme analysis will be shown in subsequent papers.

One of the greatest advantages of stable automatic analytical systems is the confidence given to evidence of minor components. A "peak" 0.005 absorbancy unit high stands out, whereas one tube in a manually read series that is increased by this amount is generally disregarded. Also very sharp peaks, which would be blurred in plots of discrete fractions, may be followed.

It is often advantageous to use a ratio recorder to distinguish between the light transmitted through the reference and the experimental cells. A recording potentiometer has been used in place of a ratio recorder in the present application because signals generated by a pH meter, conductivity bridge, and a count ratemeter or thermocouple may be conveniently reduced to a millivolt signal and recorded on the same chart.

The analytical system described was developed as a part of the Cell Fractionation Project of the Oak Ridge National Laboratory. Many of the principles are derived from the amino acid analyzer of Spackman, Stein, and Moore (5).

SUMMARY

Two systems for recording absorbancy of chromatographic effluent streams are described. The first utilizes two separate single-beam spectrophotometers and can record at any two wavelengths between 205 and 1100 mμ. The second utilizes a double-beam spectrophotometer and a pneumatic wavelength-shifting device. The latter is specifically designed for the analysis of nucleotides and nucleotide derivatives, and other compounds absorbing at 260 or 280 mμ.

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