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Analytical Techniques for Cell Fractions

XII. A Multiple-Cuvet Rotor for a New Microanalytical System[1]

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Fast automatic analytical devices are required to handle the increasing number of different analyses desired on an ever-increasing number of samples in both biochemical research and clinical studies.

Mechanized analytical systems[3] have been divided into two major groups (1). Class I systems analyze a large number of samples for a single substance or activity, whereas those of class II analyze single samples for a number of different compounds or elements. Class I systems may be further subdivided into IA, in which many samples are analyzed simultaneously, and IB, in which they are done in sequence. A variety of hybrid systems is possible, some of which have been constructed. In this paper the problem of developing analyzers of class IA is considered, and the basic studies on the development of a new analytical system are presented.

BASIC REQUIREMENTS

1. *Simultaneity.* If a series of reactions done in parallel is carried to completion, it is not important that they all be started at exactly the same time. However, many colorimetric reactions are time-

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[2] Operated by Union Carbide Corporation Nuclear Division for the U. S. Atomic Energy Commission.

[3] Automation involves feedback control of a process. Strictly speaking, nearly all analytical systems in present use are mechanized and use electronic control, sensing, and recording devices, but do not use feedback control. Hence, the words "automated" and "automation" do not apply to them despite previous use by the author and others.

dependent, e.g., with the color density increasing slowly, perhaps plateauing, and subsequently decreasing. In such instances, and where reaction rates are measured, as is the case with enzymatic reactions, it is of advantage to have all of a set of reactions start at as nearly the same time as possible, provided that all optical measurements can also be carried out over a short time span. If both the initiation of the reaction (mixing) and reading can be done in a few seconds or less, the reading may often be done very early in the course of a reaction. This is especially true when the set of samples is large enough to include a number of standards.

2. *Scaling.* The number of samples which may be analyzed with class IA analyzers should, in principle, be continuously variable. Some existing analyzers are adequate for larger loads but are not efficient for only a few (say, 1–10) samples. Small numbers of samples are usually done by manual methods. Hence, clinical and research analysts must often learn and use two different procedures for the same type of analysis, the choice depending on the volume of the analytical load. Obviously, methods that apply efficiently to both large and small sets of sample are desirable.

3. *Engagement.* If a sufficiently large number of analyses can be done in *one* apparatus and in a *short* time, the analyst can give full attention to the entire process. However, when data output is slow or a number of analyzers must be operated at the same time, the analyst's time is divided between them, with related tasks interleaved throughout the day. Because data output rate is slow, real-time computing is not economical, and data reduction is often postponed. Ideally, a set of analyses should be completed in the time required for the operator to determine whether a system of class IB is working properly. While truly automated variants which require no operator attention may ultimately be constructed, the potential for complete engagement (i.e., direct observation of reactions in real time) should still exist so that errors in dilution, reagent preparation, or procedure, or instrumental failure may be readily detected and proper action taken.

4. *Sample and reagent volumes.* Many analytical methods use enzymes and other expensive reagents. To minimize the reagent requirement, and to allow relatively small samples to be used, the techniques developed should be in the microliter range with sample and reagent volumes summing to between 0.2 and 3 ml. (It will be evident that the methods to be described are applicable to smaller or larger volumes, however.)

5. *Sample and reagent measurement.* Since discrete measurement is employed (as opposed to flow-ratio measurement and control),

the system developed should be adaptable to use with a variety of accurate volumetric devices.

6. *Sequencing of analytical elements.* If one considers each step in an analysis as an element, the following are the most common elements:

(*a*) Volume measurement.
(*b*) Measured volume transfer (two or more).
(*c*) Mixing.
(*d*) Incubation for a measured time at a known temperature.
(*e*) Precipitation (filtration or centrifugation).
(*f*) Extraction or washing of a precipitate.
(*g*) Extraction of an aqueous solution with an organic solvent.
(*h*) Absorbancy or other physical measurement, including radioactivity, pH, conductivity, etc.
(*i*) Data reduction.

The elements employed and their sequence may differ in different analyses. To be generally useful, an analytical system must be able to include most or all of these elements.

7. *Reaction monitoring.* The progress of all reactions being carried out simultaneously should be visible. A permanent chart record is not needed when reactions are carried out rapidly, if all results are monitored as they occur, and where data is reduced in real time.

8. *Data reduction.* Complete data reduction should be possible in a matter of seconds to minutes, and the computing system used should be programmed to handle both rate determinations and reactions carried to completion.

9. *Simplicity.* To be generally useful, the basic techniques must be simple, but should be applicable to a series of systems of varying complexity, ranging from a minimal-cost unit for teaching purposes to large units for handling the analytical load of a large hospital, research laboratory, or manufacturing plant.

In this and subsequent papers, the development of systems designed to fulfil these requirements is described. The present paper is concerned with demonstrating the feasibility of using a multiple-cuvet rotor to measure the absorbancies of a multiplicity of solutions simultaneously by using centrifugal force to load the cuvets, and variation in angular momentum to mix solutions.

BASIC DESIGN

The principle of double-beam spectrophotometry, in which the absorbancies of a reference solution and a sample are intercompared, either continuously or over time intervals short relative to the rate of change in intensity of the light source, is well known

To apply the same principle effectively to a larger number of samples requires that either they, or the light beam, move rapidly, one with respect to the other. We have chosen to move the samples rapidly past the light beam by using cuvet rotors. The centrifugal field inherent in this design has the advantage of providing the force for moving samples and reagents into the cuvets, for mixing them, for removing air bubbles, for sedimenting particulate matter, and for separating liquid phases. The electronic signal generated photoelectrically may be conveniently and continuously displayed on a cathode-ray tube and recorded photographically. In addition, the individual cuvet readings are made at a rate compatible with rapid electronic averaging of readings obtained over a short time period and with real-time data processing.

Cuvette Rotors. A flat 40 cuvet rotor (rotor G-I), with small, cylindrical test tubes as cuvets, was used in orienting studies. For more definitive studies, a 15 cuvet rotor (G-II) was designed. The G-II cuvets were formed by compressing a ring of $\sim$ ¼ in. Teflon sheet between 2 discs of ½ in. thick Pyrex. The Teflon section contains 15 round-bottom slots opening toward the center; the Teflon and upper Pyrex discs have a large hole in the center. Stainless-steel flanges above and below were connected with bolts to compress all parts together. A completed G-II rotor is shown in Figure 1, and a disassembled one in Figure 2. The circular apertures over the cuvets are ¼ in. in diameter, and the light beam is arranged to give a short, flat region on the end of the peaks as shown in Figure 3. When the rotor is spinning, 200 λ of solution is sufficient to fill the portion of the cuvet visible through the circular apertures in the stainless-steel end plates. The cuvet dimensions and volumes are chosen so that the rotor may be carefully brought to rest and reaccelerated without having the contents of the cuvets mix with each other when a total volume of 200–250 λ per cuvet is used. If larger volumes are to be decelerated to rest, longer cuvets must be employed.

Photoelectric sensors mounted next to the rotor edge provide synchronizing signals for the sweep circuits.

Sample and Reagent Addition. Samples and reagents are moved into the cuvets by centrifugal force. Since this may be done over a short period of time, all reactions start essentially together and may be followed continuously on the oscilloscope. Sample and reagent discs (Fig. 4) allow the sample and two reagents to be loaded into separate depressions designed not to allow fluids to mix at rest, but which all drain to the edge into the proper cuvets in a

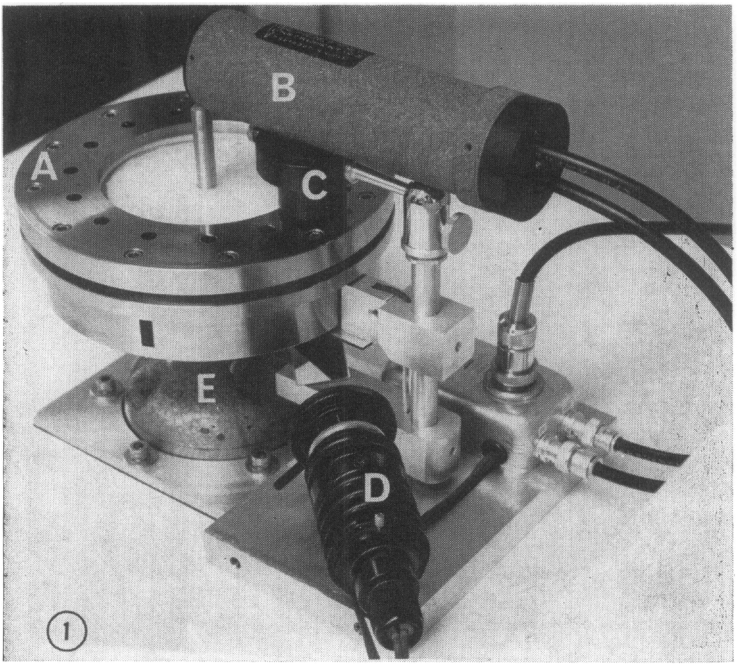

FIG. 1. G-II cuvet rotor: (A) cuvet rotor; (B) photomultiplier housing; (C) filter holder; (D) light source with diaphragm; (E) drive motor from IEC clinical centrifuge.

centrifugal field. Connections to the edge are through small capillary holes or past sloping surfaces which prevent mixing before spinning, but allow free horizontal drainage during rotation. The transfer discs may also be adapted to hold the transfer tubes previously described (1), or small, commercially available disposable microliter pipets. It is evident that these devices allow single or multiple addition reactions to be used, or reactions in which an incubation period occurs between two additions. It is also evident that precipitates formed during a reaction may be moved out of the optical path by centrifugal force, allowing the absorbancies of a clear supernatant to be measured.

Mixing. In many instances, especially where zonal centrifuge fractions are being analyzed, the reagents and the sample may differ considerably in density and viscosity. An effective means for achieving rapid mixing is therefore required.

In the cuvet rotor described, the radii to the top and the bottom of a 250 λ reaction volume differ by approximately 7 mm, giving a ratio of tangential velocities of 1.05. If the rotor is rapidly accel-

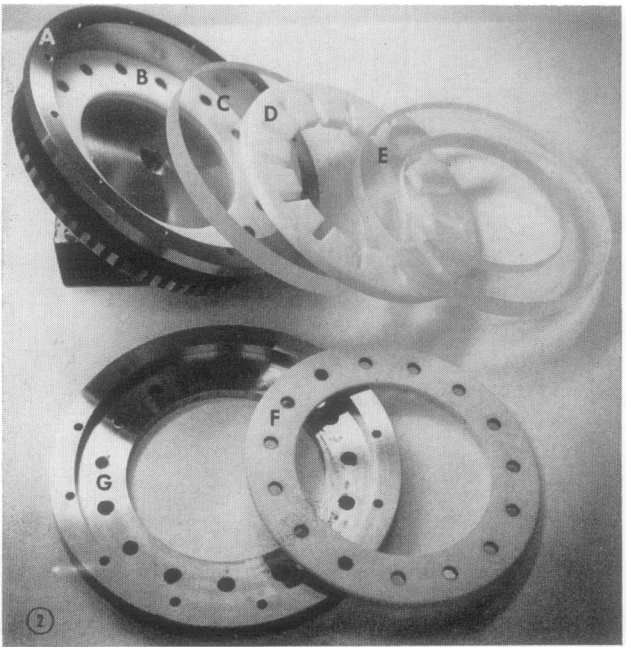

FIG. 2. Disassembled G-II rotor: (A) lower rotor housing; (B) lower gasket; (C) lower Pyrex plate; (D) Teflon cuvet spacer; (E) upper Pyrex window ring; (F) upper Teflon gasket; (G) upper stainless-steel end plate.

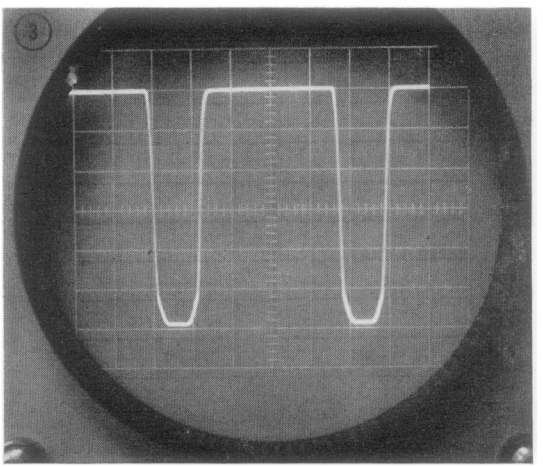

FIG. 3. Oscilloscope tracing of two peaks indicating flat tip. Baseline at top indicates 0% transmission (infinite optical density) line.

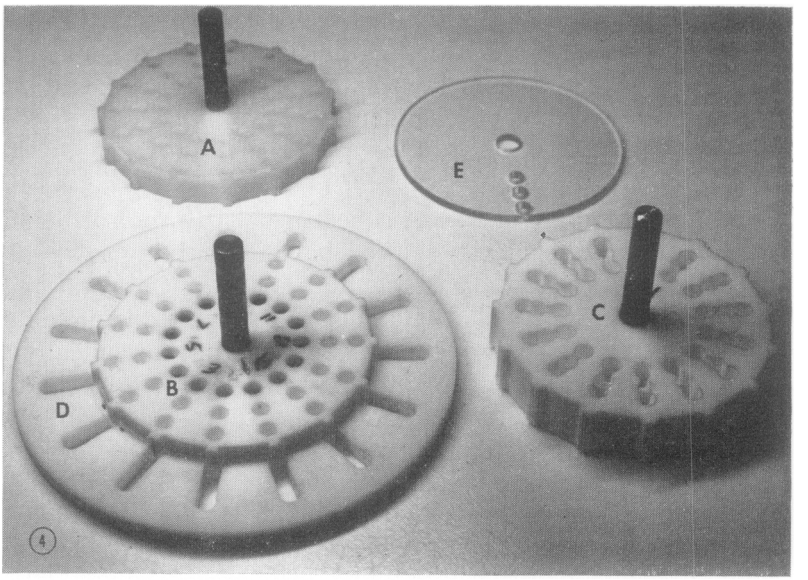

FIG. 4. Sample and reagent transfer and loading discs: (A, B) discs with cavities with top tilting toward the axis but connected by capillaries draining the bottom of the cavities to the edge; (C) disc having two concentric sets of fluid-holding cavities tilting outward so that fluid rises along cavity wall to drain through peripheral holes; (D) Teflon center piece of G-II rotor showing how loading disc fits cuvets in rotor; (E) cover plate used to cover disc and expose only cavities being filled.

erated and decelerated during fluid addition, effective circular flow is established in the cuvet because of the velocity differences between the centripetal and centrifugal surfaces of the fluid. In practice, therefore, the rotor is accelerated rapidly to transfer fluid, is rapidly decelerated (but not brought to rest), and then reaccelerated to the speed used for observation. The experiments described in subsequent sections demonstrate that adequate mixing may be obtained by this method within 10–15 sec after the rotor is started using protein samples in distilled water and a biuret reagent. With concentrated sucrose solutions, more rapid change in speed than could be effected with the present prototype will be required.[4]

[4] In subsequent studies the cuvets have been designed as syphons with a restricted entrance. Mixing may be achieved by bubbling air back through the cuvet syphon during rotation at slow speed. Detailed studies on mixing rates are included in a subsequent paper.

Cleaning. The prototypes used in these experiments have been hand-cleaned by using a fine stream of distilled water continuously removed by suction, followed by high-pressure air. An automatic cleaning device is under development.

EXPERIMENTAL

The studies recorded here are concerned with proof-of-principle and with more precise definition of problems remaining to be solved.

Leakage. Leakage from the cuvets to the edge during rotation was checked by filling the cuvets completely with water during rotation and observing the position of the fluid surface stroboscopically during rotation at 2000 rpm over an extended period. No leakage was observed. To determine whether leakage occurred between cuvets, alternate cuvets were filled with water and similarly observed. No leakage between cuvets was seen.

The possibility exists that fluid from the loading disc does not pass directly and quantitatively into the proper cuvet, but leaks laterally. A heavily stained solution of bovine serum albumin (BSA), 250 λ per cuvet, was placed in the even-numbered positions in the loading disc and moved by centrifugal force into the cuvets. The results, compared with a pattern obtained with all the cuvets full of water, are shown in Figure 5. It is seen that almost no light passed through the evennumbered cuvets, but full transmission was observed through the odd-numbered ones. The white Teflon-bottom inner surfaces of the latter cuvets were carefully examined by using stroboscopic illumination to verify that no leakage had occurred. (Note that in the oscilloscope patterns the lines connecting the peaks indicate 0% transmission or infinite absorbancy. The peaks therefore appear to be inverted when compared with those obtained with other analytical systems; however, the peak tips are all at the expected positions.)

Drainage from Loading Disc. Drainage from the loading disc was studied by using heavily stained BSA solutions. After spinning at 2000 rpm, only a very few traces of blue color could be observed in the disc. This was thought to be due to very small irregularities in the Teflon due to machining. The volumes involved appeared to be less than 1 λ. Further quantitative studies on solution transfer as a function of angular velocity, radius, disc design, and solution density, viscosity, and surface tension are indicated.

Calibration. The path length of the cuvets was measured directly by using an electronically indicating micrometer. The rotor cuvet end plates are not exactly parallel, but vary in a sine wave pattern as shown in Figure 6. This was confirmed by using identical dyed

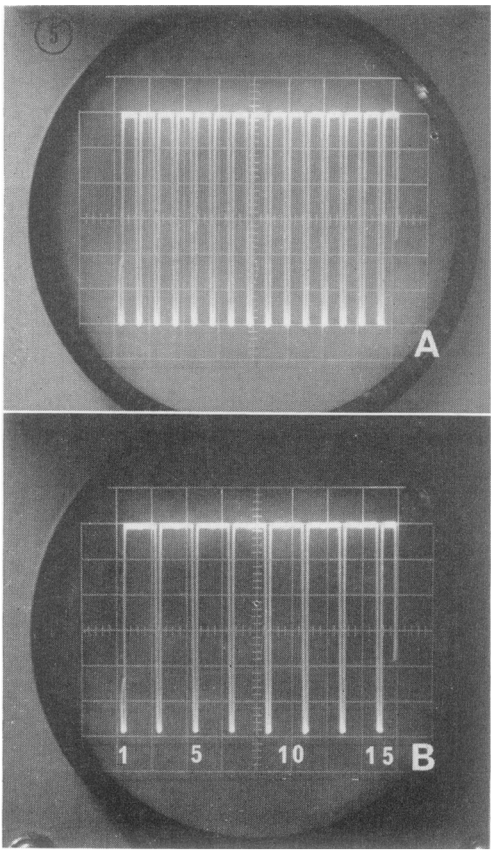

FIG. 5. Test for leakage between cuvets. (A) oscilloscope pattern with cuvets containing water. The ordinate for the tracing ranges from 0% transmission at the top to 100% transmission at the bottom of the trace. Cuvet numbers are in order from left to right. (B) Tracing seen when a solution of dyed BSA was added to even-numbered cuvets. A 550 nm interference filter was used.

protein solutions in each cuvet except the first, which contained water (Fig. 7B).

It is not desirable to depend on precision construction of cuvets to define path length or to assume that the blank (water) absorbancies of all cuvets are equal or constant. Instead blank absorbancies and absorbancies with standard solutions to determine path length should be redetermined at intervals when high precision is required. The values obtained are incorporated in the final calculations.

To see whether reporducible curves could be obtained with standard solutions, a solution containing 1.5 gm of crystalline BSA and 15 mg of bromphenol blue (BPB) in 100 ml was diluted to give

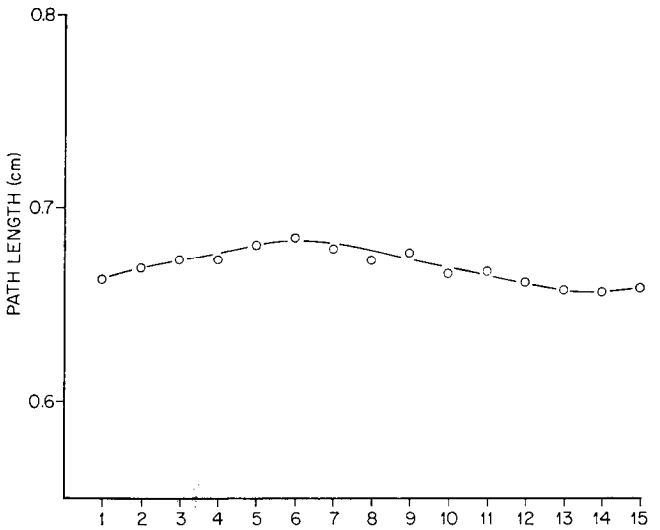

FIG. 6. Variation in cuvet path length of G-II rotor as measured with a micrometer (cuvet number on abscessa). This pattern is identical to that observed spectrophotometrically in Figure 7B. Maximum variation is ±2% of average path length.

a series of solutions containing 10% increments of the stock solution in distilled water. Figure 7A illustrates the pattern observed by using a 660 mμ filter and distilled water in all cuvets. In Figure 7B is shown the pattern obtained where a solution containing water and BSA-BPB stock 1:1 was introduced into cuvets 2–15 during rotation. The differences in peak height, although small, confirm those observed by direct measurement, as shown in Figure 6. In Figure 7C a complete series of incremented standards is shown in cuvets 3–12, with a duplicate of the stock solution used in 12 also in cuvet 14. The four remaining cuvets contained distilled water. Measurements were made on 8½ × 11 enlargements, and all peaks converted to $1/\%T$ by dividing the first blank by each subsequent reading in turn. The log of $1/T$ is the absorbance, which after blank subtraction, is then multiplied by a cuvet factor to give absorbancies for a 1 cm path length. The data obtained from Figure 7C in this manner are plotted in Figure 8.

Biuret Reaction for Protein. The experiments described thus far show that the cuvet rotor and associated electronics can be used to measure absorbancies of standard solutions, but do not demonstrate that the system can be used to follow reactions occurring in the cuvets. The biuret reaction for protein is a simple one-reagent analysis which is of general interest and is suitable for evaluation

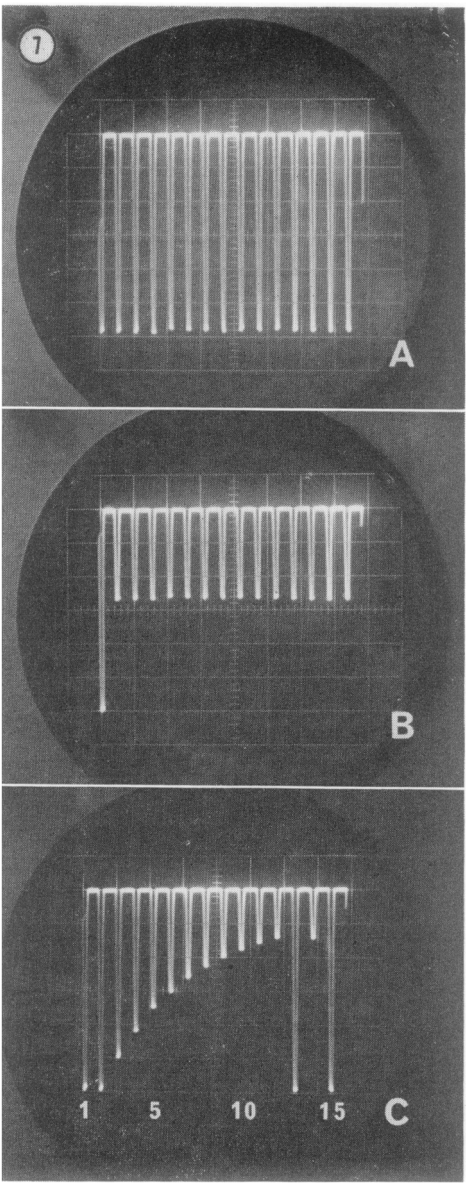

FIG. 7. Calibration curves and curves obtained with various dilutions of a dyed protein solution using 660 nm interfenrence filter: (A) all cuvets filled with distilled water; (B) cuvets 2–15 filled with a 1:1 dilution of the dyed protein stock solution; (C) dilutions of stock protein in 10% increments. Cuvets loaded as follows: water in cuvets 1, 2, 13, and 15; dilutions of stock solution in 10% increments in cuvets 3–12, with undiluted stock solutions in cuvets 12 and 14

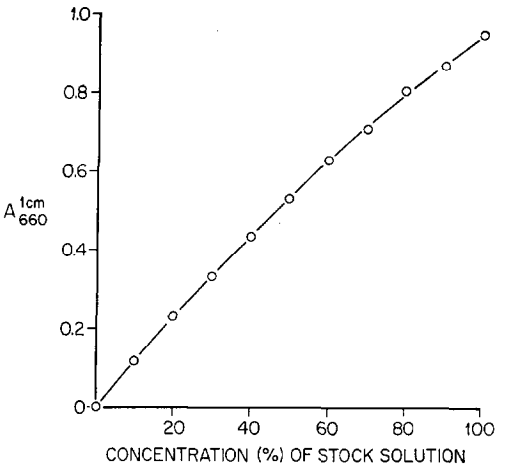

FIG. 8. Plot of results obtained in Figure 7, corrected for variation in absorbancies of water blanks and for variation in cell path length, and corrected to 1 cm light path.

of the transfer discs, of mixing, and of the technique of reading absorbancies early in the course of a reaction. The Weichselbaum biuret reagent[5] (2) may be used with protein solutions in a range of ratios varying from 0 to 50% reagent in the final mixture, provided that identical dilutions are used to obtain the standard curve.

In the experiments illustrated in Figures 9 and 10, 2 ml of various dilutions of BSA standard was mixed with 6 ml of biuret reagent and allowed to stand at room temperature 14 hr to ensure a stable color before reading. The dilutions of protein used were in increments of 0.2% protein from 0.2 to 2.0%. The results are shown in Figure 9B with water in cuvets 1, 3, 14, and 15, and the reagent blank in cuvet 2. The absorbancies, corrected to 1 cm path length, are plotted against protein concentration in Figure 10 and illustrate the results that may be expected with this system.

In the remaining studies the ratio between sample and reagent volume was changed to 1:1, and the reactions were carried out in the cuvets during rotation. Both the reagent and sample volumes were 200 λ and were introduced into the sample and reagent disc by using an automatic pipet.[6]

[5] The biurent reagent stock solution contained 45 gm sodium potassium tartrate, 15 gm copper sulfate, and 5 gm potassium iodide made up to 1 liter with 0.2 *N* sodium hydroxide. The reagent used was prepared by diluting 200 ml of this stock to 1 liter with 0.2 *N* NaOH containing 0.5% KI.

[6] Available from Baltimore Biological Laboratory, catalog No. 05-719, Baltimore, Maryland 21204.

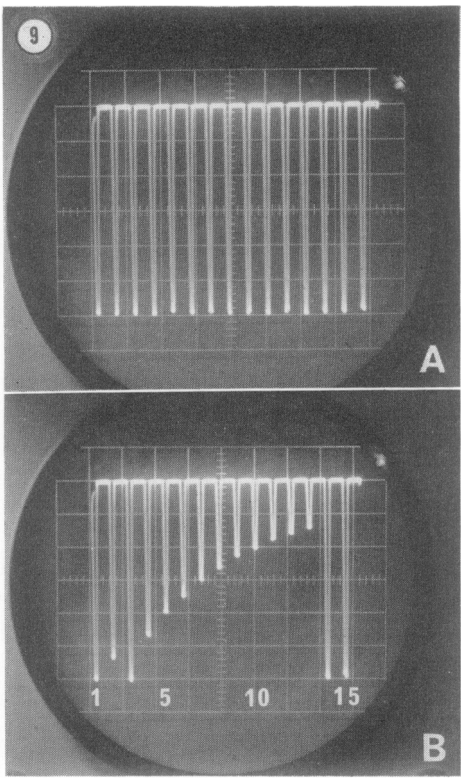

FIG. 9. Stable biuret reagent–protein standard mixtures loaded into G-II rotor with loading discs: (A) water blanks; (B) mixtures, etc. Mixtures prepared by mixing 2 ml of protein solution and 6 ml of Weichselbaum biuret reagent. Water blanks in cuvets 1, 5, 14, and 15; reagent blank in curvet 2. Series in cuvets 4–13 are prepared by using 0.2% increments of protein up to final concentration of 2.0%. All readings at 550 nm.

The reproducibility of measurements and the effect of reaction time are indicated in the two experiments shown in Figures 11 and 12. Three protein concentrations, 0.2, 0.4, and 0.6%, were used in the first experiment, the first two concentrations being run in quadruplicate, the third in pentuplicate. The pattern shown in Figure 11A was photographed 30 sec after the rotor was started, while that in Figure 11B was made after 25 min. Although a considerable increase in absorbancy is evident after 25 min, little difference in reproducibility is seen.

Figure 12 shows all the calibration data required for 30 sec protein determinations using a total of 15 blanks plus standards or samples. Water blanks are shown in Figure 12A and cuvet path comparisons obtained by using 0.6% protein standards and

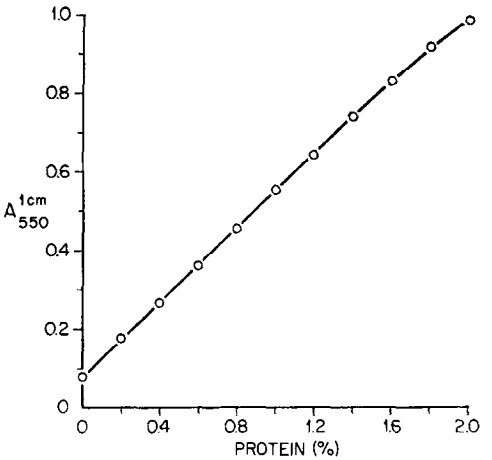

FIG. 10. Plot of data from Figure 9B corrected to 1 cm path.

one water blank are shown in Figure 12B. The series in Figure 12C was photographed 30 sec after starting the rotor (and therefore the reaction) with the sample and reagent disc loaded with water and reagent blanks, and duplicate standards having 0.2, 0.4, 0.6, 0.8, and 1.0% protein. The results are plotted in Figure 13.

DISCUSSION

The G-II cuvet rotor allows 15 reactions to be initiated simultaneously and the absorbancies to be observed and measured after very short time intervals. The rotor is an extension of the double-beam spectrophotometer concept where two absorbancies are repeatedly measured over intervals of time which are short relative to the variations in intensity that may occur in the light source.

Unlike sequential analyzers of class II, no carryover between samples is observed, and the tracing returns to 0% transmission (infinite absorbancy) between each reading. Since one or more water blanks are included in each series, readings for samples, 0, and 100% transmission are made during each revolution; thus at 1,200 rpm, 20 revolutions and 20 sets of measurements are made each second. If 1 sec exposure times are used, then the result represents the average of 20 readings. The time between peaks is ample to allow computer averaging of digitalized peak height by using the techniques described by Spragg and Goodman (3).

Errors may be due to pipetting, incomplete fluid transfer, incomplete mixing, nonlinearity in the electronics, lack of precision in measuring the photographs, and changes in the optical properties

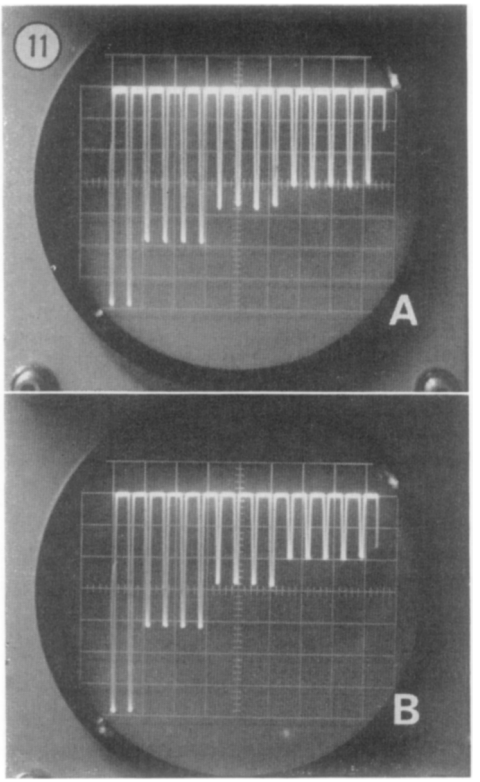

FIG. 11. Variation observed when multiple identical standards are analyzed 200 λ of protein standard or water and 200 λ of biuret solution placed on Teflon loading disc and moved into cuvets by centrifugal force. Cuvets loaded as follows: 1–2, water; 3–6, 0.2% protein; 7–10, 0.4% protein; 11–15, 0.6% protein. (A) Pattern observed 30 sec after starting rotor; (B) pattern 25 min after starting rotor.

of the cuvets during the experiment. Each of these can be examined in detail when fully automatic methods for digitalizing and printing-out results are completed, and statistical studies on the precision obtainable have been deferred until that time. It is concluded that the present studies justify completion of the entire system. It is evident that the basic concept presented here can be applied to a variety of analytical problems, especially those dependent on determination of reaction rate.

If small fluid volumes are added to the rotor initially, the rotor may be brought to a complete stop and a new sample-reagent disc inserted. In this way, reactions depending on sequential timed additions may be performed. The centrifugal capabilities of the rotor

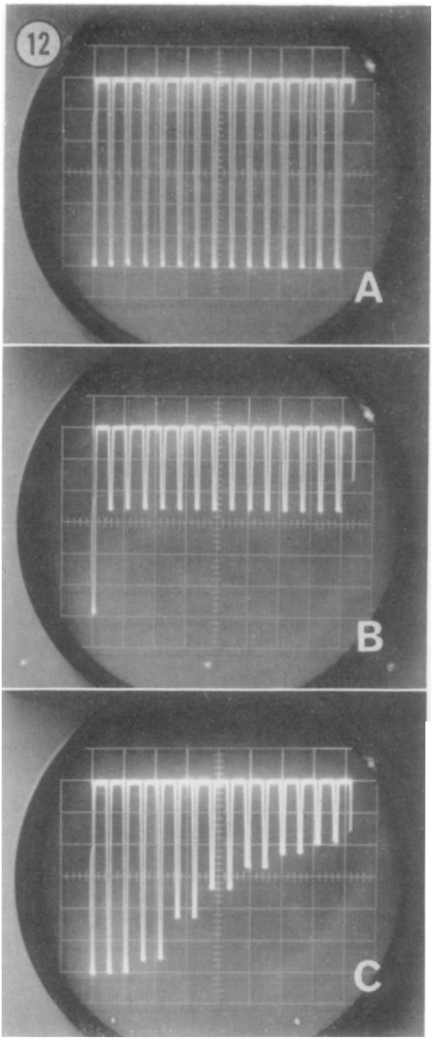

FIG. 12. Complete series for 30 sec biuret protein determination: (A) water blanks; (B) path length standardization with 1:1 mixture of biuret reagent and 0.4% BSA (read 10 min after mixing) in cuvets 2–15, and water in cuvet 1; (C) blanks and standard series read 30 sec after starting rotor. 1–3, water; 4–5, reagent blanks; 6–7, 0.2% protein; 8–9, 0.4%; 10–11, 0.6%; 12–13, 0.8%; and 14–15, 1.0%.

may also be employed to sediment particulate matter and to ensure that the solutions read are not turbid when read. In addition, the decrease in turbidity may be observed and if necessary read during centrifugation.

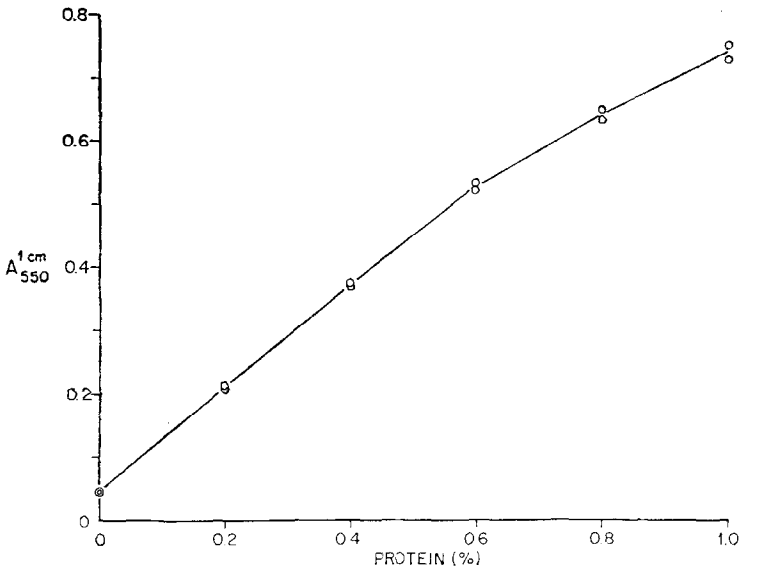

FIG. 13. Plot of data from Figure 12C corrected to 1 cm light path.

Other ways in which the rotor may be constructed include the use of removable or disposable cuvets of glass or plastic or the use of precision individual cuvets. A wide range of rotor sizes and numbers of cuvets is possible, and a number of different methods may be employed to load the sample and reagent discs. These will be explored in subsequent studies, which will also be concerned with other analytical procedures.

For the biuret reaction described to be useful for the analysis of zonal centrifuge fractions when sucrose is used as the gradient material, corrections for the color diminution produced by sucrose (4) must be made. In addition, more effective and more rapid rotor speed changes may be required to produce complete mixing with very viscous samples. Definitive studies on the biuret reaction in the G-II rotor are in progress.

More than one reaction may be carried out in one rotor at the same time. With NAD-linked assays, for example, a variety of different enzyme determinations may be done simultaneously. Decisions on how long data collection should be continued can be based on the readings obtained and the run terminated by a small computer as soon as sufficient information is obtained.

When analytical methods are employed which require incubation at elevated temperatures, the entire rotor may be heated. Standards

are always included; the temperature therefore need not be critical, provided it is uniform around the rotor. Bubbles formed by heating rapidly rise to the meniscus in the centrifugal field.

In these studies, the cuvets have been rapidly moved past a stationary light beam. The reverse situation, where the light beam scans a ring of stationary cuvets very rapidly, is also feasible.

Gradient-distributing rotors have been described (5) in which a continuous stream is distributed evenly between a series of tubes during rotation. This concept may allow reagents to be apportioned between cuvets, thus avoiding multiple pipettings.

In subsequent papers, the details of additional rotors, associated electronics, and data reduction programs, and analytical procedures will be presented.

SUMMARY

A new microanalytical system has been developed that employs a rotor containing 15 cuvets spinning past a beam of light. The signal is displayed on an oscilloscope, and a peak is observed continuously for each cuvet. Standards, samples, and reagents are placed in a central loading disc, and all solutions moved out into the cuvets by centrifugal force. Minimum volume to fill the cuvets is 200 λ. By using the Weichselbaum biuret reagent for proteins, 15 analyses may be completed in as little as 30 sec after the rotor is started. The data are obtained photographically.

Note added in proof. The General Medical Sciences-Atomic Energy Commission (GeMSAEC) system described is available in prototype form from Electro-Nucleonics, Inc., Caldwell, N. J.,; Tennecomp, Inc., Oak Ridge, Tennessee (computerized); and Union Carbide Corporation, Development Department, Tarrytown, New York.

ACKNOWLEDGMENTS

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