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Analytical Techniques for Cell Fractions

XIV. Use of Drainage Syphons in a Fast-Analyzer Cuvet-Rotor

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When a number of samples are to be analyzed by methods which involve absorbance measurements, it is of advantage to start all of the reactions at the same time and to monitor and measure the absorbancies of all of them, either continuously or at preset intervals. In a previous paper (1), a transfer disc was described that held measured volumes of samples and reagents in separate compartments so that they remained unmixed at rest. However, when the disc is placed in a GeMSAEC[3] cuvet-rotor and spun, these solutions are quantitatively transferred to their respective cuvets. The absorbancies of all cuvets were constantly displayed on an oscilloscope during rotation, and measurements made at intervals, either photographically or electronically.

A variety of problems remain to be solved before the complete system can be widely used. These problems are dealt with in this and succeeding papers. In the present study, the problem of reducing the time interval between sets of analyses is considered with the objective of reducing the interval first to approximately two minutes, and ultimately to less than one minute. The G-II rotor already described has been redesigned as G-IIB to incorporate drainage syphons that allow the cuvets to be drained, washed, and rapidly dried during rotation using air pressure. This design also allows samples and reagents to be mixed by air sucked back through the syphons during rotation. The entire G-IIB system incorporates a synchronizing circuit, a collecting ring to collect fluid

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[3] The acronym GeMSAEC has been a useful designation for the entire fast analyzer system and is derived from the major sources of support which are the National Institute of General Medical Sciences and the U. S. Atomic Energy Commission.

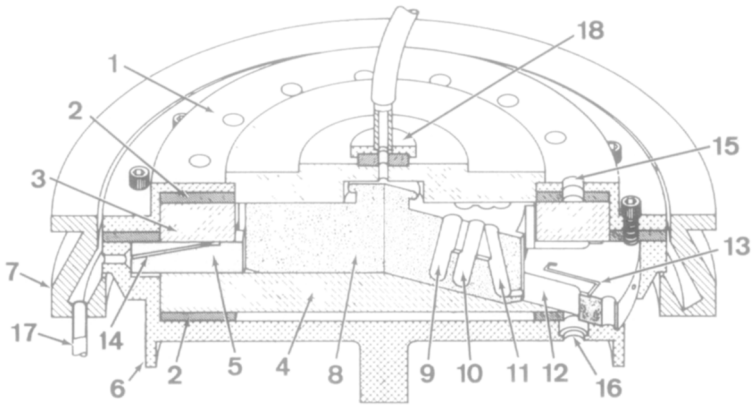

FIG. 1. Diagrammatic drawing of G-IIB rotor and transfer disc. This diagram is best understood by comparison with Figures 2–4. If the center transfer disc 8, the seal 18, and Lucite cover under it are removed, the rotor would be identical with that illustrated in Figure 3. The syphon drain line 13 and 14 in this illustration is 2 and 3 in Figure 4.

1, Upper end plate with optical ports.
2, Teflon gasket.
3, Upper annular Pyrex window.
4, Lower Pyrex disc.
5, Teflon annulus with cuvet cutout and syphon channels.
6, Lower end plate and attachment to centrifuge.
7, Stationary collecting ring to collect fluid drained from rotor.
8, Teflon transfer disc.
9–11, Interconnecting receptacles for holding sample and reagents unmixed at rest. Note peripheral port connecting to cuvet.
12, Cuvet chamber.
13, Syphon channel.
14, Outer arm of syphon channel.
15, Upper optical aperture over cuvet.
16, Lower optical aperture under cuvet.
17, Drain line in collecting ring.
18, Stationary seal over rotating seal for providing air or vacuum to rotor chamber.

drained out of the rotor, a DC power supply for the light source and photomultiplier, an oscilloscope with camera, and a variable-speed motor drive.[4]

ROTOR DESIGN

The G-IIB rotor is shown diagrammatically in Figure 1 and completely assembled in Figure 2. The rotor is similar in over-all dimensions to the G-II rotor previously described (1), with the following modifications.

The center chamber enclosing the transfer disc is covered with a plastic disc containing a rotating seal surface in its center. A stainless-steel

[4] Adapted from a GLC centrifuge manufactured by Ivan-Sorvall, Inc., Norwalk, Conn.

static seal with a small pressurizing spring to keep the sealing surfaces in contact is held by a cross-bar that can be easily swung to one side.

The cuvet chambers were modified as shown in Figures 3 and 4 to allow efficient centrifugal drainage. The centrifugal portion of the cuvet (rounded end) is tilted 80° from horizontal toward the syphon. Approximately 300 μl of liquid is required to fill the cuvets so that all light through the 1/4 in. diameter aperture passes through liquid. When 600 μl is centrifuged in each cuvet, the syphons do not drain, whereas 700 μl will produce automatic draining without added air pressure. The path length through the cuvet is 1 cm.

TRANSFER DISC OPERATION

The Teflon transfer disc employed in these studies is shown in side section in Figure 1. It is evident that a great variety of configurations may be employed to hold two or more small fluid volumes unmixed at rest, but to allow them to flow centrifugally through one or more aper-

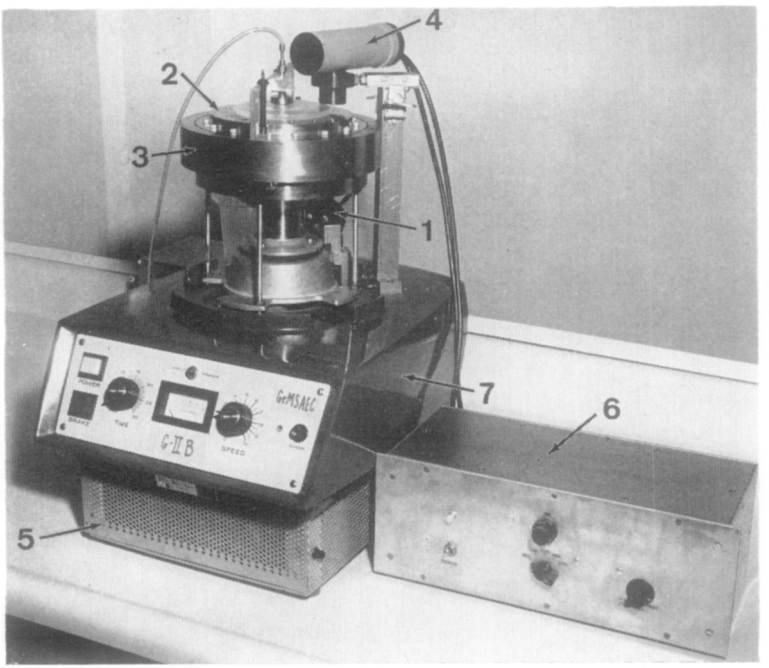

FIG. 2. Completely assembled G-IIB analytical system:

1, Light source.
2, Rotor.
3, Collecting ring.
4, Photomultiplier housing.
5, Sweep circuit synchronizer.
6, Photomultiplier and light source power supply.
7, Centrifuge drive.

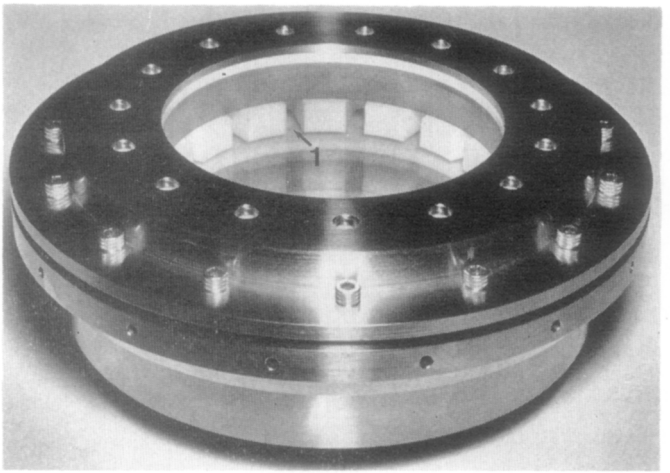

FIG. 3. Assembled G-IIB rotor showing cuvet 1 opening to rotor center.

tures or ports into the cuvets of the cuvet-rotor during acceleration. Each ring of recessed depressions in the transfer disc is called a register.

Speed of Transfer. The first question to be asked is: do the fluids in each member of a register transfer fluid simultaneously? For rapid reactions it is important that all reactions in a set start at as nearly the same time as possible.

Visual observation of the oscilloscope indicates that transfer of sufficient liquid to fill the cuvets past the optical aperture level occurs during an interval of less than one second during acceleration, and at approximately 350 rpm. Acceleration is rapidly continued, however, since

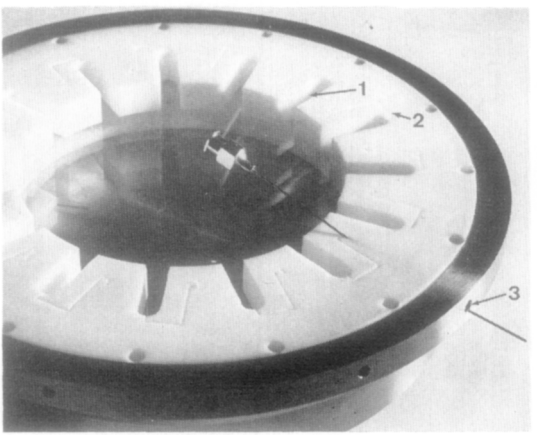

FIG. 4. Partially disassembled G-IIB rotor showing: 1, Cuvets. 2, Syphon channels. 3, Exit port for outer syphon limb (illustrated with needle).

examination of the transfer disc after acceleration to 500 rpm revealed small droplets remaining in the disc.

Efficiency of Transfer. The efficiency of transfer as a function of maximum speed attained was next examined. Using an automatic micropipet,[5] 200 μl was placed in each hole in the first (closest to the axis) and second register. The weight of the water was determined gravimetrically and was usually within 1% of the expected volume as shown in Table I. After acceleration to a given speed and rapid deceleration to rest, the disc was again weighed to determine the amount of water remaining in the disc. A Lucite cover was used during this experiment to prevent loss by evaporation. Inspection of the results in Table 1 indicates that transfer is incomplete at 500 rpm, but is in excess of 99% above 1000 rpm. The

TABLE 1
Transfer of Water from Transfer Disc as a Function of Rotor Speed[a]

Max. rotor speed, rpm	Wt. of water before acceleration, gm	Wt. of water remaining in disc, gm	Water not transferred, %
2000	6.08	0.00	0.0
2000	6.04	0.03	0.5
1500	6.04	0.05	0.8
1000	6.00	0.06	1.0
500	6.04	0.37	6.1
500	6.04	0.32	5.3

[a] Thirty 200-μl samples placed in disc in each experiment.

drive used with the G-IIB rotor accelerates from 350 (initial transfer speed) to 1000 rpm in 2.5 seconds. Transfer therefore occurs in bulk within 1 second for approximately 90% of the liquid, and the remainder is transferred within 2.5 seconds.

MIXING

Considerable mixing occurs during transfer because the liquids flow sequentially through the same passageways and chambers in the transfer disc, move laterally during flow into the cuvet due to Coriolis forces and rotor acceleration, and tend to flow down one side producing a rotating motion in the liquid in the cuvet. Mixing is opposed by the stabilizing effect of centrifugal force, however, which can be quite effective if two liquids having quite different density are employed. With the G-IIB rotor, air can be drawn back through the syphons to produce mixing. With

[5] Micropipet available from Baltimore Biological Supply Company, Baltimore, Md.

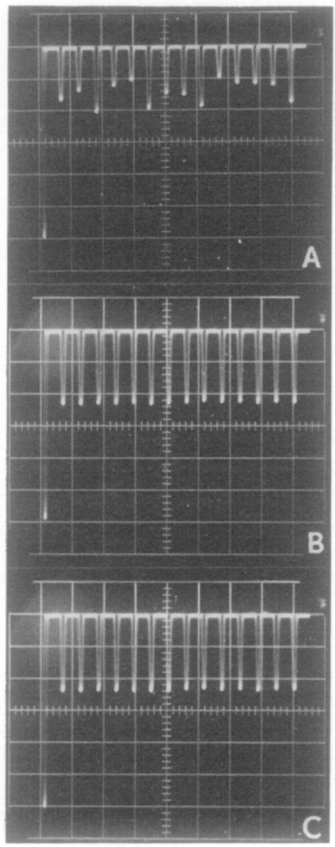

FIG. 5. Demonstration of rapid mixing by suction during rotation. Number 1 cuvet contains water in each instance. 200 μl of 30% sucrose and 200 μl of a protein-biuret reagent mixture (unmixed) placed in remaining cuvets. A, Oscilloscope absorbance pattern (550 nm). B, Same as A but after 2 sec mixing by air sucked back through syphons. C, Same after an additional 5 sec of suction.

volumes of less than 0.6 ml this can be very effective, and in addition can withdraw liquid in the syphon to mix with that in the cuvet.

To examine mixing experimentally, 200 μl of each of two solutions was used per cuvet. The first was 30% sucrose in water (density 1.125 gm/cc at 25°C) and the second was a mixture of the biuret reagent previously described (1) and an equal volume of 0.8% bovine serum albumin in water. A 550 nm interference filter was used on the photomultiplier. The sucrose solution was placed in the third register, the biuret mixture in the first register, except for the register chambers for cuvet number 1 which both contained water. The rotor was rapidly accelerated to 1000 rpm, and then decelerated to 400 rpm and the pattern photographed (Fig. 5A).

It is evident that incomplete mixing had occurred. A vacuum line attached to the seal was opened for 2 seconds, and the pattern immediately photographed (Fig. 5B). Movement of air through the cuvets could be readily observed on the oscilloscope, giving visual indication that mixing was occurring in all cuvets simultaneously. To determine whether mixing was indeed complete, the vacuum line was opened for an additional period of 5 seconds (Fig. 5C). No change was noted, indicating that 2 seconds was sufficient to produce complete mixing. In additional experiments 1 second of air movement was found sufficient to produce complete mixing under identical conditions. To determine the shortest mixing time required (which will be in the millisecond range), special timing devices will be needed.

Since some mixing may have occurred during radial flow in the transfer disc and flow down the cuvet wall, these experiments were repeated transferring the sucrose to the cuvet-rotor using one transfer disc, and then transferring the lighter biuret-protein mixture as a separate step using a second transfer disc to encourage layering. Mixing for one or two seconds was as effective under these conditions as in the previous experiment.

SYPHON OPERATION

The prime requirement is that drainage occur when desired with great reliability. In a centrifugal field, syphon operation may be erratic because a negative pressure sufficient to draw liquid inward in the drain line against centrifugal force is not produced. This may occur when a small air bubble is left at the high point of the syphon drain line or when fluid flows out of the syphon along one side of the drain line allowing air to move in a reverse direction. These problems are minimized by making the drain line diameter small. However, if the line is too small it may be clogged more easily.

The solution to this problem is to use a positive method for draining the cuvets, in this instance by air pressure introduced at the rotor center. Fluid drained from the rotor is collected in a ring (Fig. 1) surrounding the rotor.

One obvious defect of the present design is that part of the liquid introduced into the cuvet initially flows at once into the syphon and is imprisoned there. If the sample and reagent (or reagents) have not been uniformly mixed before this occurs, the precision of the analysis will be adversely affected. As previously mentioned, this source of error is minimized by drawing air back through the syphon and through the liquid in the cuvets.

Since all of the steps during the course of an analysis are followed in all cuvets in real time on the oscilloscope, failure of a syphon to drain, cuvet leakage, or incomplete mixing may be detected at once.

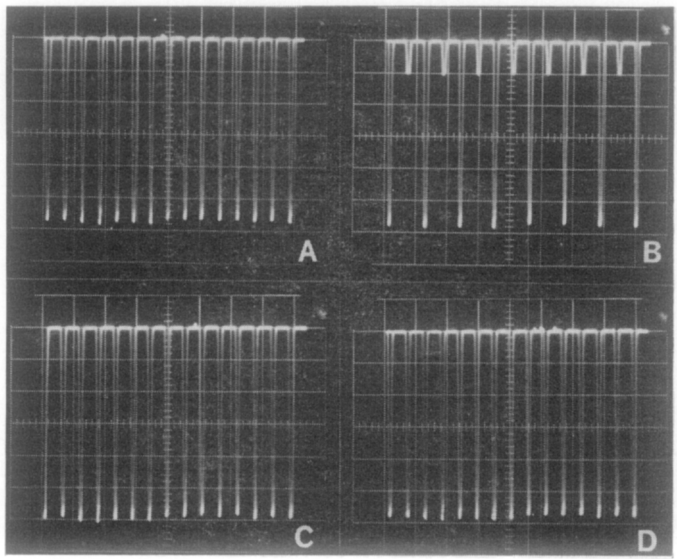

FIG. 6. Demonstration of syphon draining of G-IIB rotor: A, Oscilloscope pattern showing water blanks. B, Pattern with protein-biuret mixture in even-numbered cuvets. C, Pattern after drainage of cuvets under air pressure during rotation, and reloading with 400 μl of water in each cuvet; comparison with A shows negligible carryover in even-numbered tubes. D, Pattern after draining second time and refilling with 400 μl of water per cuvet; comparison of A, C, and D shows that more than 99% of the cuvet volume is drained through the syphons under air pressure. As noted in the previous paper (1), the oscilloscope pattern represents the per cent transmission on a linear vertical scale for each of the fifteen cuvets simultaneously. The line along the top represents zero transmission or infinite optical density.

Reproducible syphon drainage was obtained at 500 rpm using air pressure. The maximum pressure is required at the last moment of drainage and is directly proportional to the square of the rotor speed. Calculated maximum pressure is 0.38 psig at 500 rpm and 1.5 psig at 1000 rpm. Slightly higher pressures were required in practice because of seal leakage.

Experimentally, drainage under pressure is accomplished at 500 rpm, followed by rapid acceleration to 1000 rpm to drain small droplets into the syphon line, followed by deceleration to rest under air pressure. This procedure requires approximately five seconds using manual braking of the rotor.

WASHING OF CUVETS

The amount of carryover from one set of analyses to the next depends on the amount of solution left in the syphon and cuvets after drainage, and on the number and volume of washes employed. When sequential

sets of analyses are performed using the same procedure, the possibility of eliminating washing may be considered if less than 1% of the reaction volume remains after drainage.

To measure the residuum after drainage, the following technique was employed. A photograph of the oscilloscope pattern was obtained using 400 μl water in each cuvet (Fig. 6A). The rotor was then emptied and dried with air. A transfer disc was then loaded with water and biuret-protein mixture (400 μl per cuvet) alternating and rapidly accelerated to 1000 rpm giving the pattern shown in Figure 6B. The rotor was then drained using air pressure at 500 rpm, immediately accelerated to 1000 rpm, and braked to rest under air pressure. A transfer disc containing 200 μl of water in each chamber of the first and second register was then inserted and the rotor accelerated to 1000 rpm. To mix any residual liquid in the syphon with the water, suction was applied at 500 rpm. The results are shown in Figure 6C. The water was then drained under air pressure and a second set of water samples transferred into the rotor giving the pattern shown in Figure 6D. Comparison of patterns A, C, and D of Figure 6 shows that less than 1% of the biuret-protein mixture remained in the cuvet. Measurement of seven cuvets in a separate experiment showed an average of 0.2% remained.

In the rotor used, the connection for air and suction is made through a cover in the center of the rotor. The same ends may be served by using a hollow drive shaft and a rotating seal at its lower end, or the syphon drain lines may be brought toward the center axis underneath and connected to a suitable seal.

SEALING THE ROTOR

In the G-II and G-IIB rotors, the cuvets are formed by compressing an annulus of Teflon between a disc and an annulus of Pyrex glass. Teflon will cold flow very slowly under pressure and gradually form a flat, polished surface quite distinct from the "orange peel" surface ordinarily observed. However, it is very difficult to tighten the screws holding the end pressure plates together in such a manner that the glass does not crack before the Teflon is deformed sufficiently to give flat parallel surfaces. The softness of Teflon makes it unlikely that precision flat and parallel surfaces can be formed initially by machining. A method for gradually applying a uniform pressure was therefore sought.

The coefficients of expansion of the rotor components are shown in Table 2. It is evident that Teflon expands more than either glass or stainless steel as the temperature is raised. Temperature cycling may therefore be used to make the Teflon components conform exactly to the stainless steel and glass surfaces. The rotor was assembled and gently tightened,

TABLE 2
Thermal Expansion Coefficients

Material	cm/cm/°C
Teflon	99×10^{-6}
304 stainless steel	14.4×10^{-6}
Pyrex	3.3×10^{-6}

using a torque wrench, and a torque of 5 inch pounds. The rotor was then heated to approximately 200°C and allowed to cool to room temperature. The assembly screws were then found to be loose, i.e., the Teflon had expanded, been slightly compressed, and had shrunk back on cooling to smaller dimensions. The screws were retightened and the process was repeated. The temperature used is well below the melting point of Teflon, but is sufficient to soften it somewhat, facilitating compression forming. After two such cycles with tightening with a torque of 5 inch pounds, the screws were again tightened, this time to 10 inch pounds, and the rotor tested for leakage using the method previously described (1).

DISCUSSION

A combination of a three-register transfer disc and a fifteen-cuvet rotor with small syphons attached has been used to explore methods for accelerating the rate at which analyses may be performed. From oscilloscopic observation of liquid transfer and measurements of the liquid remaining in the transfer disc, it is concluded that approximately 90% of the liquid is transferred to the cuvets in a period of less than 1 second, and that over 99% is transferred when 1000 rpm was reached 2–3 seconds later. It is concluded that transfer is sufficiently uniform to consider that all reactions in one analytical set proceed in parallel for all practical considerations.

Mixing by sucking air back through the drain syphons attached to the cuvets was extremely rapid. Using 30% sucrose and a protein-biuret reagent mixture, complete mixing was observed in 1 second.

From these studies it is evident that rapid fluid transfer and mixing can be accomplished in the G-IIB rotor. Should even faster initiation be required, it is evident that a much faster acceleration rate can be obtained using more powerful drives to both complete transfer and mixing in 1 second or less. We conclude that transfer and mixing times have been reduced to the point at which they constitute a negligible fraction of the total analysis time.

Draining and washing of the original cuvet-rotor occupied several minutes. Using the drainage syphons, the rotor may be drained of more than 99% of the reaction mixture in a few seconds. Flushing with water

once can easily be done in 30 additional seconds. With more rapid acceleration and braking, these times could be further reduced. In addition, modifications of the transfer disc are being fabricated which allow the wash water to be added as soon as the reaction mixture is drained, further reducing the washing interval. A turn-around time of 1 minute is therefore practical.

A heat cycle method for pressure-forming the Teflon cuvet-spacer is described which facilitates fabrication of cuvet-rotors.

With the improvements incorporated in the G-IIB rotor, the limiting factors in the development of fast analyzers now become (*a*) the time required for the wet chemical procedures used, and (*b*) the time required to measure samples and reagents into transfer discs. Methods for reducing these intervals are discussed in subsequent papers, together with analytical procedures specifically adapted to the G-IIB system.

SUMMARY

A cuvet-rotor, designated G-IIB, has been developed that contains small-bore syphons attached to each of the fifteen cuvets. Drainage of reactants from the transfer disc was found to be approximately 90% complete in 1 second and 99% complete after 2.5 seconds during acceleration to 1000 rpm. Mixing of protein-biuret reagent solution and 30% sucrose occurred in 1 second when a vacuum was used to pull air back through the syphons and cuvets. Drainage of the cuvets through the syphons was almost quantitative (>99%) when air pressure and one cycle from 400 rpm to 1000 rpm and back to 400 rpm was used. The interval between successive sets of analyses may therefore be 1 minute or less. A method of heat cycling to make the Telflon cuvet-spacer conform to the glass windows is presented.

REFERENCE

1. Anderson, N. G., Analytical techniques for cell fractions. XII. A multiple cuvet-rotor for a new microanalytical system, *Anal. Biochem.* **28**, 545 (1969).