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Analytical Techniques for Cell Fractions

IX. Measurement and Transfer of Small Fluid Volumes[1]

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The zonal centrifuge systems previously described (1, 2) yield a large number of fractions, each sufficiently large to allow many different analyses to be made. This is only feasible with automated or semiautomated analytical systems (3–5). We are therefore interested in developing such systems for elemental, specific compound, and enzyme assay.

Automated analyzers may be divided into two broad classes. Class I systems analyze a large number of samples for a single substance or activity and may be termed multisample, single-component analyzers. Systems of class II analyze one sample at a time for a large number of different substances or activities and may be termed single-sample, multicomponent analyzers. The chromatographic systems previously described (6, 7) are in class II. Hybrid systems have been devised in which a series of samples are chromatographed sequentially. The initial problem with analyzers for zonal centrifuge fractions is to develop a series of systems of class I.

Class I analyzers may be further subdivided into those using discrete sampling and volumetric measurement of reagents (class IA) and those using controlled rates of sample and reagent flow to achieve the desired ratio of sample and reagents (class IB) (8). Analyzers of the latter type have been used for protein (9) and enzyme analysis (3–5) of fractions from the A-IX, B-IV, and B-XV zonal rotors. The viscosity of the denser portion of the gradient and difficulties in achieving good mixing with concentrated sucrose samples have been sources of error. Analyzers of

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class IA would offer certain advantages if the problems of accurate measurement, transfer, and mixing of small volumes could be overcome.

A large number of micropipetting devices have been described for volumes of less than 1 ml (10, 11), including self-filling pipets and those which are filled to a mark by visual inspection. It is unlikely that mechanization of devices originally devised for human hands will solve the problem. Rather, measuring systems specifically adapted for automation are needed. The requirements are as follows:

1. The volume-measuring space must be reproducibly defined.
2. Air bubbles in the sample, or air in any part of the system, must not lead to inaccuracies.
3. Volume measuring must be independent of the density, viscosity, and surface tension of the fluids measured.
4. Cross-contamination of samples must not occur.
5. Transfer of fluid from the measuring device to a mixing or reaction chamber must be quantitative.
6. Precision should be at least $\pm 0.5\%$.
7. The mechanism for measurement and transfer should be usable in the 30–1000 μl range.
8. The techniques used should allow a reasonable number of simultaneous measurements or transfers.

Devices depending on suction to fill a defined volume suffer from inaccuracies that arise from (*1*) air bubbles, (*2*) difficulties in meniscus reading or accurate plunger withdrawal, (*3*) differences in the viscosity, density, or surface tensions of samples, and (*4*) unless rinsing or disposable pipets are used, cross-contamination. An additional source of error is variation in the amount of fluid pendant on the tip of the pipet.

To avoid these problems we have reasoned as follows. First, the volume-measuring container should be small and disposable. It follows then that we must ask what sorts of vessels can be made easily and very reproducibly of plastic. If the interior of the measuring chamber is defined by a multicomponent die, the die parts may not always be positioned in exactly the same way, and varying amounts of plastic may protrude into die divisions. It appears that the space defined by a single polished pin as shown diagrammatically in Figure 1 is the most reproducible cavity obtainable in plastic.

To fill the tubes completely, means must be found for removing even very tiny air bubbles from the inside. In a centrifugal field, the buoyancy of an air bubble is proportional to the centrifugal force (neglecting pressure since it is not great in short tubes). Air bubbles do not persist in

ordinary aqueous solutions at several hundred times gravity. We conclude that use of centrifugal force eliminates error due to air bubbles in these tubes. (A variety of ways for arranging to fill large numbers of tubes and of draining away excess fluid outside the tubes are described in a subsequent paper.)

When the fluid around a centrifugally filled tube is removed, a convex meniscus remains. The curvature of the meniscus is a function of the surface tension and density of the fluid. As noted by Kirk (10), small

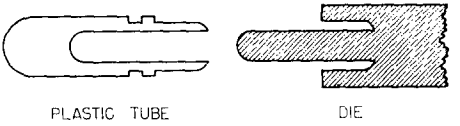

FIG. 1. Schematic drawing of measuring tube and die.

volumetric flasks resembling test tubes with a scribe mark are extremely inaccurate because the meniscus cannot be well defined since its shape is dependent on the physical properties of the solution and the contact angle. We require a method for producing a reproducible flat meniscus.

A fluid protruding from a small hole in a nonwetting plastic is similar in many respects to a drop on a flat surface of the same material. The maximum height such a drop can have is given by the equation (see ref. 12):

$$h^2 = \frac{2\gamma}{g\rho} \tag{1}$$

where h = height in cm, γ = surface tension in dynes/cm, g = 980 dynes, and ρ = density of the liquid. It is evident that the height is inversely proportional to the square root of the gravitational force and may be very considerably flattened in a centrifugal field. The force should be chosen so that variations in meniscus curvature produced by differences in surface tension are negligible, and so that a given tube always contains an identical volume after the second centrifugation. Equation 1 gives the maximum meniscus height. With tubes of small diameter, the actual height would be much less.

The problem of quantitative transfer remains. Small fluid droplets must not be allowed to remain in the measuring vessel, even with solutions of high viscosity. This, again, is best achieved by using centrifugal force.

We recognize that these techniques are *unhandy*. They are, however, well adapted to mechanization. In this they resemble binary counting devices, which, taken singly, are of little practical use, but which have unique properties when properly arrayed in large numbers.

EXPERIMENTAL

We ask only whether the methods outlined will yield identical volumes and quantitative transfers.

For initial studies, plastic components have been machined to reduce costs. Characteristics of molded tubes will be discussed in another paper.

The sequence of steps used experimentally is shown in Figure 2. After centrifugal filling, the tubes were capped and weighed. They were then

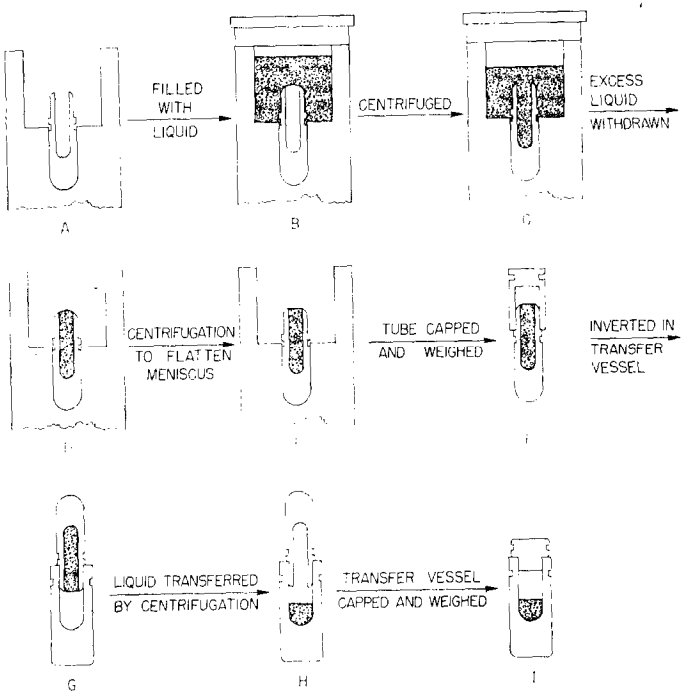

FIG. 2. Schematic diagram of procedures used to determine reproducibility of filling and transfer method.

uncapped and inserted in the transfer vessels and centrifuged. The tubes were then removed from the transfer vessels, and the latter capped and weighed.

The radius from the centrifuge axis to the meniscus in the measuring tube (Fig. 2D) was 11 cm, whereas the radius to the top of the transfer vessel was 18 cm. All centrifugal procedures were performed in the International PR-2 centrifuge at room temperature (25°C). The centrifuge was accelerated rapidly to speed in each instance and turned off as soon as speed was reached. Tubes were filled at 2000 rpm (490 *g* at top of measuring tube); menisci were leveled at 2500 rpm (769 *g* at meniscus); fluids were transferred at 2000 rpm (805 *g* at top of transfer vessel).

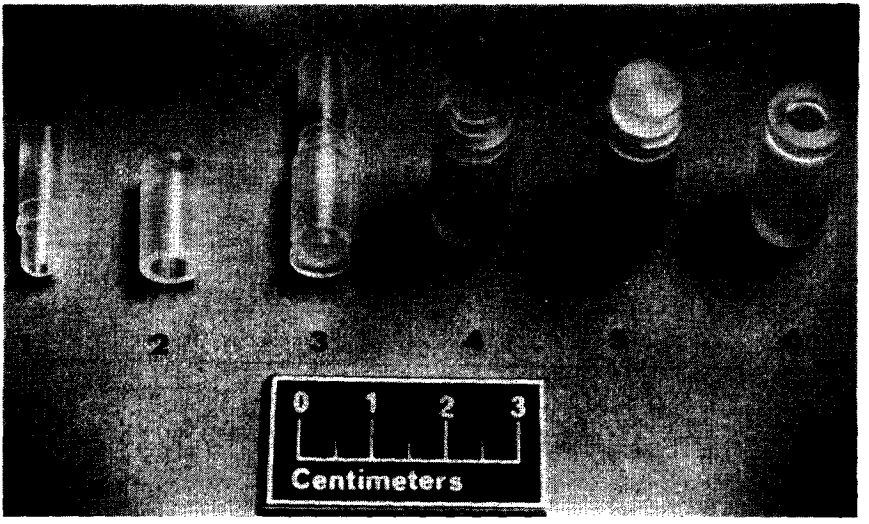

FIG. 3. Plastic measuring and transfer containers: (1) large plastic measuring tube (~120 μl); (2) cap for measuring tube; (3) measuring tube with cap in place; (4) measuring tube inverted into transfer vessel; (5) transfer vessel with cap; and (6) transfer vessel without cap.

Two measuring tubes with similar external dimensions were used. Both had chambers $\frac{5}{8}$ in. deep; diameters were either $\frac{1}{8}$ or $\frac{1}{16}$ in. The tubes, transfer vessels, centrifuge cup adapters, and caps are shown in Figure 3. Kel-F (a fluorinated hydrocarbon) and nylon measuring tubes were used, and all transfer vessels were of Kel-F.

By using equation 1, the maximum height of a water droplet at 770 g would be 139 μ. This is less than 1% of the height of the liquid column. Variations in meniscus curvature produced by differences in fluid density or surface tension, therefore, would be only a small fraction of 1% of the volume.

The results obtained with pure water in Kel-F and nylon tubes are shown in Table 1. With three different Kel-F tubes, standard deviations of 0.67, 0.31, and 0.28% were observed. The nylon tube gave a standard deviation of 0.69%. Three smaller (~30 μl) tubes gave standard deviations on replicate filling of 0.31, 0.22, and 0.28%, whereas the single nylon tube had a standard deviation of 0.67%. In general, nylon tubes have been inferior to Kel-F tubes.

In the centrifugal transfer studies, the average losses in four series of experiments with the large tubes were 0.0, 0.2, and 0.2 mg for Kel-F and 0.8 for nylon. With the smaller tubes, average losses were 0.2, 0.1, and 0.05 mg in three sets of experiments with Kel-F tubes, and 0.3 mg with a nylon measuring tube.

TABLE 1

Weight of Distilled Water in Measuring Tubes and After Transfer to a Receiving Vessel [a]

Tube No.	Results with 1/8 X 5/8" Chamber		
	Wt. of water in tube, mg	Wt. of water in transfer vessel, mg	Difference mg
1 Kel-F	122.0	122.3	+ 0.3
	120.4	120.4	0.0
	122.0	122.2	+ 0.2
	122.5	122.3	- 0.2
	122.7	122.4	- 0.3
	122.3	122.3	0.0
	Av = 122.0	Av = 122.0	Av loss = 0.0 mg
	SD = 0.82 mg (0.67%) (V = 122.2 µl)	SD = 0.78 mg (0.64%) (V = 122.2 µl)	
2 Kel-F	123.6	123.5	- 0.1
	123.2	123.0	- 0.2
	123.1	122.9	- 0.2
	124.1	123.8	- 0.3
	123.5	123.4	- 0.1
	Av = 123.5	Av = 123.3	Av loss = 0.2 mg
	SD = 0.39 mg (0.31%) (V = 123.7 µl)	SD = 0.37 mg (0.30%) (V = 123.5 µl)	

Additional experiments were performed with a 0.05% solution of sodium dodecyl sulfate and with 45.1% sucrose as shown in Table 2. The detergent solution density was not appreciably different from water and yielded very similar results. With sucrose, slightly more variation was seen than with water alone. Volumes are calculated by using a density of 0.9981 for water and 1.2048 (13) for 45.1% sucrose at 23.5°C. A summary of the results is shown in Table 3. It is evident that errors in

TABLE 1 (Continued)

3 Kel-F	121.3	120.9	- 0.4
	120.9	121.1	+ 0.2
	121.0	120.6	- 0.4
	121.5	121.4	- 0.1
	121.7	121.2	- 0.5
	Av = 121.3	Av = 121.0	Av loss = 0.2 mg
	SD = 0.34 mg (0.28%)	SD = 0.31 mg (0.26%)	
	(V = 121.5 µl)	(V = 121.3 µl)	
4 Nylon	125.0	123.8	- 1.2
	124.4	123.6	- 0.9
	124.3	123.6	- 0.7
	126.1	125.8	- 0.3
	123.9	123.2	- 0.7
	Av = 124.7	Av = 124.0	Av loss = 0.8 mg
	SD = 0.86 mg (0.69%)	SD = 1.03 mg (0.83%)	
	(V = 124.9 µl)	(V = 124.2 µl)	

Results with 1/16 X 5/8" Chamber

5 Kel-F	32.2	32.0	- 0.2
	32.0	31.8	- 0.2
	32.1	31.8	- 0.3
	32.0	31.9	- 0.1
	32.0	31.9	- 0.1
	Av = 32.1 mg	Av = 31.9	Av loss = 0.2 mg
	SD = 0.10 mg (0.31%)	SD = 0.09 mg (0.28%)	
	(V = 32.1 µl)	(V = 31.9 µl)	
6 Kel-F	32.2	32.1	- 0.1
	32.2	32.2	0.0
	32.1	32.0	- 0.1
	32.1	32.0	- 0.1
	32.2	32.2	0.0
	Av = 32.2	Av = 32.1	Av loss = 0.1 mg
	SD = 0.07 mg (0.22%)	SD = 0.10 mg (0.31%)	
	(V = 32.2 µl)	(V = 32.2 µl)	

TABLE 1 (Continued)

7 Kel-F	31.7	31.7	0.0
	31.8	31.8	0.0
	31.9	31.8	- 0.1
	31.8	31.7	- 0.1
	31.9	31.9	0.0
	Av = 31.8	Av = 31.8	Av loss = 0.04 mg
	SD = 0.09 mg (0.28%)	SD = 0.16 mg (0.50%)	
	(V = 31.9 µl)	(V = 32.0 µl)	
8 Nylon	31.5	30.8	- 0.7
	31.4	31.0	- 0.4
	31.1	30.8	- 0.3
	31.0	30.9	- 0.1
	31.2	31.0	- 0.2
	Av = 31.2	Av = 30.9	Av loss = 0.3 mg
	SD = 0.21 mg (0.67%)	SD = 0.10 mg (0.32%)	
	(V = 31.3 µl)	(V = 31.0 µl)	

[a] In all tables: SD = standard deviation; V = volume; Av = average.

measurement and loss in transfer have been kept within the desired range of 1%.

DISCUSSION

A method for measuring accurately and for quantitatively transferring small fluid volumes has been developed. All previously available methods working in the microliter range have been calibrated either to contain or to deliver a specified volume. The "to contain" pipets must be rinsed several times to achieve quantitative transfer. Delivery pipets are subject to rather large errors when solutions differing in density, viscosity, and surface tension are used. The method described here is independent of these factors over a rather wide range and is well adapted to automated manipulation. In addition to measuring volumes, the method is well adapted to micropycnometry. The plastic used must be inert, nonwetting, and dimensionally stable, and should not take up moisture. Kel-F has

TABLE 2

Measurement of Weight of Solutions in Kel-F Measuring Tubes and Weight of Solution after Transfer

Tube No.	0.05% sodium lauryl sulfate	
	Wt. in measuring tube, mg	Wt. in transfer vessel, mg
1	[a]	122.5
	122.0	122.0
	122.5	122.5
	122.0	122.0
	Av = 122.17	Av = 122.25
	SD = 0.29 mg (0.23%)	SD = 0.29 mg (0.24%)
	(V = 122.4 µl)	(V = 122.5 µl)
5	31.9	31.7
	31.9	31.8
	31.9	31.9
	31.9	31.5
	Av = 31.9	Av = 31.725
	SD = 0.00 mg (0.00%)	SD = 0.17 (0.53%)
	(V = 32.0 µl)	(V = 31.8 µl)

[a] Transferred immediately without weighing.

been found suitable for this purpose; indication of water absorption by nylon renders nylon unsatisfactory.

When centrifugal force is employed to fill simple plastic tubes, to level menisci, and to transfer fluids, the average error between consecutive measurements and the loss on transfer is well below 1%. These errors may be due largely to errors in weighing (since a balance sensitive to only 0.1 mg was used), to evaporation, and to the fact that the ratio between diameter and length of the measuring space was kept large to simplify fabrication.

TABLE 2 (Continued)

6	32.1	32.0
	32.0	32.0
	32.1	31.9
	32.1	31.8
	Av = 32.07	Av = 31.92
	SD = 0.04 mg (0.12%)	SD = 0.09 mg (0.28%)
	(V = 32.3 µl)	(V = 32.0 µl)
7	[a]	31.9
	31.7	31.7
	31.7	31.7
	31.9	31.7
	Av = 31.77	Av = 31.75
	SD = 0.12 mg (0.38%)	SD = 0.15 mg (0.47%)
	(V = 31.8 µl)	(V = 31.8 µl)

[a] Transferred immediately without weighing.

In addition, no special precautions were taken to control temperature, and all components were manipulated by hand since we are interested in evaluating the technique under the conditions in which it may be used. The errors observed are well below the 1% level required.

The ratio between the diameter and depth of the masuring tubes has not been examined. These ratios were 1:5 and 1:10 in the tubes tested. Additional work will be required before the optimal ratio is known.

With higher centrifugal fields and with much smaller vessels, it is evident that measurement and transfer below the microliter range should be possible, providing the problem of evaporation can be solved.

In subsequent papers the application of the measuring and transfer concepts presented here to both manual and automated analyses will be presented.

SUMMARY

A method for measuring and transferring small fluid volumes has been developed. The measuring devices are small Kel-F tubes which are filled

TABLE 2 (Continued)

	45.1% Sucrose w/w	
1	147.5	147.4
	147.2	147.1
	147.2	147.2
	Av = 147.3	Av = 147.23
	SD = 0.17 mg (0.11%)	SD = 0.15 mg (0.10%)
	(V = 122.3 µl)	(V = 122.3 µl)
2	148.4	148.5
	147.9	147.9
	147.9	148.0
	Av = 148.07	Av = 148.13
	SD = 0.29 mg (0.20%)	SD = 0.34 mg (0.23%)
	(V = 122.9 µl)	(V = 122.9 µl)
5	38.5	38.2
	38.2	38.2
	38.4	38.2
	Av = 38.37	Av = 38.2
	SD = 0.15 mg (0.39%)	SD = 0.0 mg (0.00%)
	(V = 31.8 µl)	(V = 31.7 µl)
6	38.6	38.6
	38.5	38.5
	38.6	38.5
	Av = 38.57	Av = 38.53
	SD = 0.06 mg (0.15%)	SD = 0.06 mg (0.16%)
	(V = 32.0 µl)	(V = 32.0 µl)

and the meniscus flattened by centrifugal force. The measured volume is then transferred quantitatively by centrifugal force. The reproducibility is well below 1%. The method is independent of surface tension, air bubbles, or viscosity over a wide range.

TABLE 3

Volumes in Microliters Calculated as Described in Text
(Averages From Experiments in Tables 1 and 2)

Tube	H_2O		Sodium Dodecyl Sulfate		45.1% Sucrose	
No.	Measured	Transferred	Measured	Transferred	Measured	Transferred
1	122.2	122.2	122.4	122.5	122.3	122.2
2	123.7	123.5	———	———	122.9	122.9
3	121.5	121.3	———	———	———	———
4[a]	124.9	124.2	———	———	———	———
5	32.1	31.9	32.0	31.9	31.8	31.7
6	32.2	32.2	32.1	32.0	32.0	32.0
7	31.9	32.0	31.8	31.8	———	———
8[a]	31.3	31.0	———	———	———	———

[a]Measuring tubes of Nylon, all others made of Kel-F.

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