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*AN IMPROVED MAGNETIC DENSITOMETER: THE PARTIAL SPECIFIC VOLUME OF RIBONUCLEASE**

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This communication describes an increase in precision of the magnetic densitometer for small volumes[1] and presents results of partial specific volume determinations on ribonuclease.

Because of the importance of the partial specific volume in characterizing a macromolecule, a need exists for a method of making rapid, accurate density measurements on small volumes. Conventional pycnometric techniques and the moving, magnetically controlled buoy of Lamb and Lee[2] and MacInnes *et al.*[3] are sufficiently accurate when large amounts of material are available; for most biological macromolecules, however, it is difficult and often impossible to obtain adequate amounts in high purity. Density gradient columns of immiscible liquids,

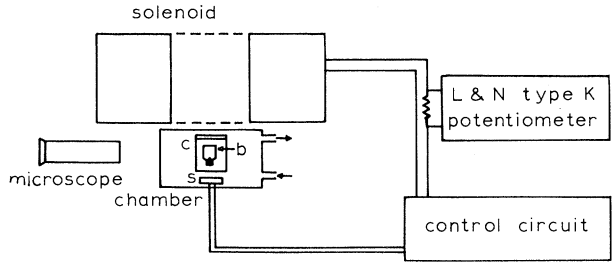

FIG. 1.—Schematic diagram of magnetic densitometer components. (*b*) Buoy; (*c*) glass cell containing sample liquid and buoy in thermostated water bath; (*s*) sensing or Q-coil.

on the other hand, employ very small amounts of material, but this method requires considerable time and skill, and it is difficult to rule out the possibility of solvent exchange or changes in solution structure. Current improvements in the magnetic densitometer now permit a routine sensitivity of $\pm 2 \times 10^{-6}$ gm/ml and a precision of $\pm 1 \times 10^{-5}$ gm/ml on 300 μl of solution with 10 min required per measurement. These characteristics make this instrument well suited to the study of the partial specific volume of proteins and other macromolecules.

A schematic diagram of the magnetic densitometer is shown in Figure 1. The buoy (*b*) is pictured as levitated in the cell (*c*) which contains the liquid sample. As the buoy falls because of gravitational pull, the impedance of the sensing or Q-coil (*s*) increases. Via the electronics circuit described previously,[1] the solenoid current is increased commensurately so that a stable support of the buoy is maintained. The solenoid current required to suspend a buoy is a function of the position of the buoy and the buoyant force or solution density. A finely engraved reference line on the buoy is observed through a rigidly mounted, 20-power micro-

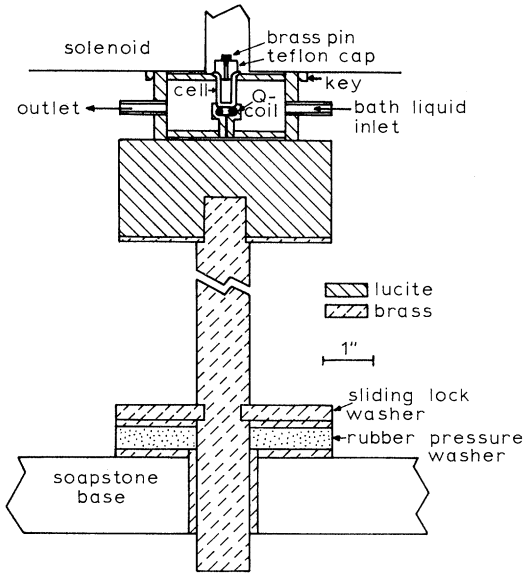

FIG. 2.—Constant temperature cell and mount.

scope with reticule lines enabling the buoy to be repositioned within 3 μ. With the buoy always suspended at the same height, the solenoid current is a function only of the solution density. The current is determined by measuring the potential across a manganin wire resistor of 6.2 ohms with a potentiometer which was readily estimated to 10^{-5} v. The constant temperature cell is held firmly against the bottom of the solenoid by a stand as shown in Figure 2. The response of the electronic circuit is 280 ma for each millimeter of vertical motion of the buoy. Since the measured fluctuation in the support current is about 1 μa, the over-all vertical motion of the buoy is less than 10^{-6} cm.

When the opposing forces on the suspended buoy are equalized, it can be shown that the sensitivity is given by

$$\frac{dI}{d\rho} = \frac{-I}{2(\rho_B - \rho)}, \tag{1}$$

where I is the solenoid current, ρ the solution density, and ρ_B the density of the buoy. Hence, the sensitivity can be increased by making the density of the buoy close to that of the solution. Buoys made of a "Kel F" exterior and HYMU "80" (Carpenter Steel Co.) interior, as shown in Figure 3, have been very satisfactory. The magnetic material must exhibit negligible hysteresis and magnetic memory; upon annealing in a hydrogen atmosphere, the HYMU 80 meets the requirement for this application. Kel F has the favorable property of expanding at nearly the same rate as water at 20°; in the experiments reported, the temperature varied in a range of 0.02° which would affect the apparent density less than 10^{-6} gm/ml. The

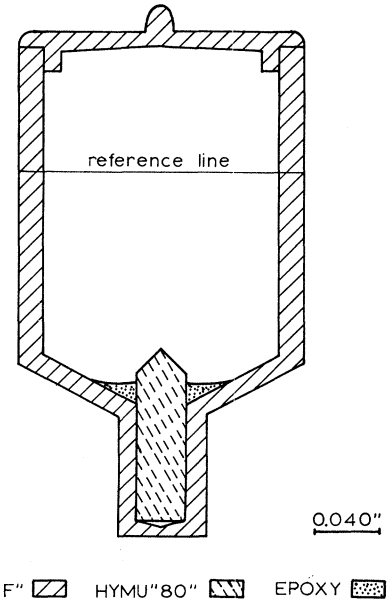

FIG. 3.—Cross section of magnetically controlled buoy. Dissymmetry in the buoy allows it to rotate when levitated until the same segment of the reference line is presented to the field of view.

volume of this buoy is 0.8 cm^3 with a density of 1.020 gm/cm^3. Epoxy resin was used to adjust the buoy density and seal the cap.

Calibration, relating support current to solution density, was achieved through the use of National Bureau of Standards sucrose (lot 6004) and tables.[4] A least-squares fit was made to the available data in this table from 0 to 4 per cent concentrations; no point differed from the fit by more than four parts per million. The empirical equation thus obtained was used to compute the density of the sucrose solutions of known concentration prepared by us. For this purpose the sucrose was ground to a powder in an all-glass mortar and pestle, dried *in vacuo* over activated alumina at room temperature, and weighed into the appropriate weight of glass-distilled water on a balance reading to 0.005 mg. The calibration data is shown in Table 1. In every determination the difference between the least-squares reference density and the observed density was five parts per million or less. For a well-equilibrated cell, the solution can be changed and the potentiometer reading repeated to $\pm$ 20 μv. Hence, for a first degree coefficient of 0.07 gm/ml/v, the corresponding uncertainty in density reading is about $\pm 2 \times 10^{-6}$ gm/ml. To check the calibration, the density of recrystallized, pulverized and dried, reagent grade potassium chloride was measured and compared with the value computed from the International Critical Tables[5] at 20°. Table 2 shows these results. From

TABLE 1

CALIBRATION OF BUOY AT 20° IN SUCROSE

Concentration* (gm/100 gm solution)	$(I_0 - I)$ (10^{-5} v)	Reference density† (gm/ml)	Observed density‡ (gm/ml)	Difference (10^{-6} gm/ml)
0	0	0.998234	0.998327	3
0.541443	3168	1.000335	1.000336	1
0.986539	5944	1.002064	1.002063	−1
1.465760	9148	1.003929	1.003925	−4
1.894768	12258	1.005604	1.005600	−4
2.601022	18062	1.008369	1.008374	5
3.008976	21951	1.009971	1.009976	5
3.597066	28756	1.012287	1.012284	−3

* National Bureau of Standards sucrose lot 6004 in glass-distilled water.
† The least-squares fit to the data in column 3 from reference 4 is $\rho = 0.998238 + 0.386734\ C + 0.106241\ C^2$ where C is the weight fraction.
‡ A least-squares fit of the densities computed from the fitted line of column 3 is $\rho = 0.998237 + 0.068410\ (I_0 - I) - 0.068028\ (I_0 - I)^2$.

TABLE 2

DENSITY OF KCL SOLUTIONS AT 20°

Concentration (gm/100 gm solution)	Reference density* (gm/ml)	Observed density (gm/ml)
0.47145	1.00126	1.00125
1.14807	1.00560	1.00560
1.43606	1.00745	1.00741

* Computed from a first-order fit of the data in reference 5 between 0 and 4% concentration.

the internal agreement of these calibration studies we conclude that the magnetic densitometer has a precision of 1 part in 10^5.

For the studies on ribonuclease-A, detailed measurements as a function of concentration were performed on different lots of chromatographically purified preparations from Sigma Chemical Company and from Worthington Biochemical Corporation. All samples were determined at 20° in 0.15 *M* KCl; except for one series, small amounts of phosphate[6] or glycine were added to maintain the pH. The samples were not specially deionized.

Experimental.—For each density-concentration series, approximately 50 mg of a protein sample was dissolved in about 2.5 ml of the chosen solvent and dialyzed in a size 18/32 Visking cellophane bag versus 300 ml of the solvent for about 24 hr; the solvent was changed at least once during the interim. The final protein concentrations were from 15 to 30% lower than that estimated from the weighed-in amounts, but moisture content of the samples, adsorption to the cellophane, and volume changes during dialysis probably account for most of the apparent loss because the nitrogen content of the dialyzates agreed with that of the undialyzed solvent within experimental error. Also, the densities of the dialyzate and pure solvent were identical. The refractive increment of the protein solution at 20° versus dialyzate was determined in a differential refractometer at 546 mμ. The instrument was calibrated frequently with Bureau of Standards sucrose (lot 6004) and all such results fell within a 0.5% range throughout this study. Dilutions of this stock solution with dialyzate were made using recalibrated constriction micropipets (Carlsberg type); the volume of each diluted solution was between 600 and 800 μl. The precision of delivery was ascertained by weighing to be within 0.3 μl. The diluted solutions were kept in the cold in thoroughly cleaned and dried vials which were tightly capped until density measurements were made (usually within 12 hr). Following the density measurements, most of the diluted solutions were pooled and redialyzed against the same solvent, the refractive increment was determined, and duplicate samples, containing 12–20 mg protein, were taken for dry-weight analysis. Comparable volumes of dialyzate were also dried. The solutions were evaporated at 60–70° and final drying was achieved in an open dessicator over activated alumina in an oven maintained at 106°. After 24 hr, the weighing bottles were recapped, the dessicator was sealed, and the entire unit was removed from the oven to cool to room temperature. The weighing bottles were then removed and weighed, still sealed, on a balance reading to 0.005 mg. In this way the possible rapid uptake of moisture by dried protein from the room air upon opening a dessicator was minimized; the same change in buoyancy for weighing bottles containing dried protein and dialyzate was assumed. The operation was repeated until constant weight (within 0.1 mg) was attained; 3–5 alternate heating and weighing cycles were performed on each dried sample. The material in each weighing bottle was then dispersed with concentrated sulfuric acid at 100° and quantitatively transferred to volumetric flasks for nitrogen analysis by a Kjehldahl-type procedure.

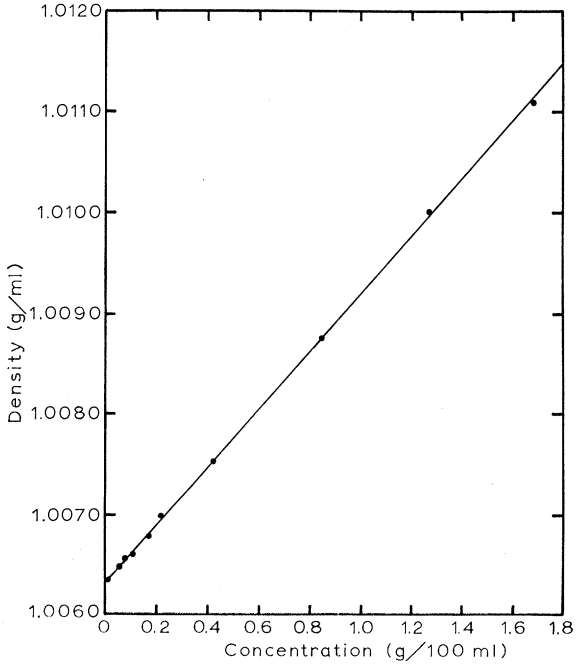

FIG. 4.—Density versus protein concentration for Sigma, Type III, ribonuclease-A in 0.15 *M* KCl—0.007 *M* (K) phosphate, pH 7.6. The solid line is a least-squares fit to the experimental points.

The specific refractive increments, $\Delta n/c$, for all ribonuclease samples were within $\pm 1.5\%$ of a mean value of 0.1919 ml /gm.[7] Although the values for some of the samples tended more toward one extreme than the other from this mean, several additional determinations would have been required to substantiate a statement of real differences. The agreement in dry weights between duplicate samples and between duplicate dialyzates was usually within 0.5%, yielding an over-all uncertainty in the difference value (protein) for the duplicate pairs of <1%. Repeated dry-weight analyses for a given lot number increased the uncertainty to about 1.5%. Our precision with refractive measurements, however, is 0.5%. Accordingly, we have chosen to assess the concentration of all stock solutions by refractive measurements using the mean value of 0.1919 ml/gm for $\Delta n/c$. In this way, values of $\bar{v}$ observed for different preparations more accurately reflect real differences in the materials as received, but are possibly less accurate on an absolute basis.

Results.—A plot of density versus concentration from one stock solution of Sigma, Type III, ribonuclease-A is shown in Figure 4. The slope from a least-squares fit to the data yields $\bar{v}$ according to the relation

$$\bar{v} = \frac{1}{\rho_0}\left[1 - \left(\frac{d\rho}{dc}\right)_{c\to 0}\right], \tag{2}$$

where ρ_0 is the density at infinite dilution of the protein and c is grams protein per milliliter. A similar number of concentrations were used for each result listed in Table 3, and all plots intercepted the density value of the dialyzate at the ordinate.

TABLE 3

THE PARTIAL SPECIFIC VOLUME OF RIBONUCLEASE-A SAMPLES

Sample*	$\bar{v}$ (ml/gm)
0.15 *M* KCl—0.007 *M* Phosphate (K), pH 7.6	
Sigma, Type III, lot R-22B-70 (N = 17.0 ± 0.2%)	0.7060 ± 0.0006
	0.7046 ± 0.0016
	0.7040 ± 0.0019
	0.7036 ± 0.0008
Sigma, Type III-A, lot 43B-772-3, (N = 16.9 ± 0.2%)	0.6979 ± 0.0011
	0.6955 ± 0.0012
	0.6988 ± 0.0006
Worthington (PO_4 free), lot RAF 6060 (N = 16.4 ± 0.2%)	0.7004 ± 0.0010
	0.7030 ± 0.0010
Worthington (PO_4 free), lot RAF 6065 (N = 16.3 ± 0.2%)	0.7005 ± 0.0013
Worthington (PO_4 free), lot RAF 6067 (N = 16.5 ± 0.2%)	0.7134 ± 0.0006
0.15 *M* KCl—0.010 *M* Glycine (K), pH 9.6	
Sigma, Type III-A, lot 43B-772-3	0.6919 ± 0.0017
	0.6923 ± 0.0020
0.15 *M* KCl, pH 7.8	
Sigma, Type III-A, lot 43B-772-3	0.6949 ± 0.0015

* The range of protein concentration over which densities were determined for computing each value of $\bar{v}$ was 1.52 ± 0.18% to 0.07 ± 0.02%.

The standard error of each ρ versus c slope is reflected in the value listed for $\bar{v}$. Although our experience shows that most determinations can be made within an uncertainty of ±0.001 ml/gm, the lower precision of the concentration measurements precludes assigning a reliability within 0.003 ml/gm (a difference of 1 per cent in the concentration results in approximately 0.003 ml/gm change in $\bar{v}$). Further, it must be emphasized that the concentration of protein is based upon our definition of the dry weight which is not necessarily the true mass of the anhydrous material.

The results for the different preparations, in some instances, are substantially outside the range of experimental error as deduced from the degree of internal consistency shown for two of the samples (Sigma Types III and III-A). There was

no close correlation with the number of enzyme activity units listed by the suppliers. We conclude with McMeekin[9] that the different values reported in the past for this protein are, at least to a large extent, a result of differences in the preparations. In this connection it is pertinent to note that value for $\bar{v}$ obtained on the same lot of Sigma Type III ribonuclease by Reithel and Sakura[10] at 25° using a density-gradient technique is in excellent agreement when consideration is given to differences in temperature, solvent, and assessment of concentration. The rather small range of values among the different preparations in Table 3 compared to the range of values in the older literature no doubt attests to the progress in purification of this protein.

Phosphate ion apparently binds to ribonuclease extensively and lowers the isoelectric point. At low concentrations of phosphate, the isoelectric point is about 7.6–7.8, whereas in the presence of some univalent buffer species the isoelectric point is about 9.5.[11] In addition to the KCl medium at pH 7.6 in dilute phosphate, results in the lower part of Table 3 are reported also for the Sigma Type III-A ribonuclease-A at pH 9.6 in the presence of glycine. These results show a small, but apparently real, decrease in $\bar{v}$ (about 0.006 ml/gm) at the higher pH. Since the protein was presumed to be isoelectric in either medium, a significant difference was unexpected. According to the findings of Rasper and Kauzmann,[12] approximately 5 moles of hydroxyl ion are taken up per mole of ribonuclease in going from pH 7.6 to 9.6 in an unbuffered solution of similar ionic strength to ours. Their results by dilatometry in this pH range show a volume increase of about 80 ml/mole of protein, or 0.0058 ml/gm. A density series was carried out, therefore, in the absence of phosphate at the lower pH in 0.15 *M* KCl. The solution was dialyzed under a nitrogen atmosphere and the pH was adjusted with KOH. The final pH of the dialyzate was virtually the same (7.8) as that of the stock solution containing 1.34 per cent ribonuclease. As seen at the bottom of the table, the value for $\bar{v}$ was still slightly higher (approximately 0.003 ml/gm) than that at pH 9.6. Assuming no binding by the glycinate ion, we cannot account for the negative solvent increment except to reiterate that none of the preparations we used were put through a mixed-bed ion exchange procedure to remove possible bound ions in the material. We have since noted *both* higher and lower values of $\bar{v}$ at pH 9.6 than at 7.6 with other commercial preparations of ribonuclease, again pointing up differences in the packaged products.

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*TWO SEQUENTIAL REPRESSIONS OF DNA SYNTHESIS IN THE ESTABLISHMENT OF LYSOGENY BY PHAGE P22 AND ITS MUTANTS**

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Wild-type phage P22 (hereafter designated c^+) is a temperate phage. On infection of its host, *Salmonella typhimurium*, some cells may lyse, but others survive as lysogenic progeny. Lysogenic bacteria are characterized by the ability to produce the infecting phage, but are immune to subsequent infection by the phage. The controlling element of the lysogenic condition is the prophage, which is the phage genome integrated into the bacterial hereditary material.[1-3]

The c^+ phage produces turbid plaques owing to the growth of lysogenic cells in the plaque center. A larger number of clear-plaque-forming mutants, affected in the ability to become prophage, have been isolated. These fall into three complementation groups: c_1, c_2, and c_3.[4] The class c_3 mutants are still temperate, but give lower frequencies of lysogenization than does c^+. The clear mutants c_1 and c_2 behave like virulent phages, since they lyse all infected cells with liberation of progeny particles. In mixed infection with mutants of the c_2 type, phages of the c_1 class lysogenize as well as the c^+ phage. Genetic studies place these three groups of mutants into three closely linked, but nonoverlapping, complex loci.[5] The complementation exhibited by the mutants of these different cistrons suggests that a number of phage-controlled functions are necessary for the establishment of lysogeny.

This report describes studies of the rate of incorporation of tritiated thymidine into infected cells during the establishment of lysogeny. In experiments with c_1 and c_2 phage, deviations from the pattern of incorporation exhibited by c^+-infected cells allow conclusions as to the functions of the c_1 and c_2 loci. If the rate of H^3-thymidine incorporation is equated to the rate of DNA synthesis, the data may be interpreted as showing that these loci control two sequential repressions of DNA synthesis in infected cells, both repressions being necessary for lysogenization.

Materials and Methods.—Bacteriophage strains: Purified stocks of wild-type (c^+) phage P22 and two complementing clear mutants, c_1^7 and c_2^5, were used in these experiments. A complete description of the characteristics of these mutants and their positions on the phage linkage map has been given.[4, 5]

Bacterial strains: Salmonella typhimurium strain LT2 was used for all infections. A *Gal*– mutant was used as background for plating.