The Simultaneous Determination of Partial Specific Volumes and Molecular Weights with Microgram Quantities* †

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S. J. Edelstein§ and H. K. Schachman
From the Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720

SUMMARY

A method is described for the simultaneous measurement of partial specific volumes and molecular weights of proteins and other substances by sedimentation equilibrium experiments in H₂O and D₂O (or D₂O¹⁸) solutions. In effect, the method is a differential sedimentation equilibrium technique based upon the change produced in the equilibrium concentration distribution when the density of the solution is increased by the use of D₂O (or D₂O¹⁸). The equations used for the two parallel sedimentation equilibrium experiments in H₂O and D₂O (or in H₂O and D₂O¹⁸) account for deuterium exchange by the incorporation of a factor which changes only slightly for different proteins and can be estimated with sufficient accuracy from knowledge of the composition of the solute. Experiments were conducted on H₂O and D₂O solutions of adenosine, ferredoxin, ribonuclease, myoglobin, α-chymotrypsinogen, and bovine plasma albumin, and the resulting values of the partial specific volumes and molecular weights were in excellent agreement with currently accepted values. In addition, excellent results were obtained for myoglobin and α-chymotrypsinogen from parallel sedimentation equilibrium experiments on H₂O and D₂O¹⁸ solutions. With D₂O¹⁸ solutions the potential accuracy in the determination of partial specific volumes is substantially greater than with D₂O since the density of D₂O¹⁸ is so much greater and the consequent effect on the redistribution of the solute molecules is so large. The method is direct and no more laborious than a conventional sedimentation equilibrium experiment, does not depend on knowledge of the solute concentration, yields partial specific volumes accurate to better than 1.0%, and can be used in certain situations with impure samples.

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† The authors have indicated that their desire to use the symbol D₂O¹⁸ with the superscript following the chemical symbol rather than preceding it is based on the hope that ambiguity in reading the compound would be reduced thereby. The Journal is making an exception to the usual rule concerning isotope notation in the present instance; however, this is not to be considered as a general precedent.

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All ultracentrifugal methods for determining molecular weights are dependent on knowledge of the partial specific volume, \( \bar{\epsilon} \), of the substance of interest (1). Despite this requirement, experimental values of \( \bar{\epsilon} \) are not available or, alternatively, the measured \( \bar{\epsilon} \) is not of the desired accuracy. For proteins, in particular, reliable values for \( \bar{\epsilon} \) are necessary since errors in the evaluation of this quantity are magnified about 3-fold in the subsequent calculation of molecular weights (2). This occurs inevitably because \( \bar{\epsilon} \) for proteins is about 0.74 ml per g and the term appearing in all the basic equations has the form \((1 - \bar{\epsilon}p)\), where \( p \) is the density of the solution (generally 1.0 g per ml).

Although presently available ultracentrifugal techniques in which the photoelectric scanning absorption optical system (3) is used yield the requisite sedimentation equilibrium data with only microgram quantities of protein, a compatible method for the determination of \( \bar{\epsilon} \) with similar amounts of material has not been described heretofore. Standard procedures for the evaluation of \( \bar{\epsilon} \) require orders of magnitude more protein either for density measurements, as in pycnometry (4), or for determination of the dry weight concentrations, when density gradient columns (5) or magnetic floats (6-9) are used. The estimation of \( \bar{\epsilon} \) from amino acid composition (10) also requires at least 0.5 mg of protein for the amino acid analysis and is not directly applicable for metalloproteins and proteins containing other prosthetic groups.

In order to eliminate the requirements and limitations of the standard techniques a method has been developed for the determination of \( \bar{\epsilon} \) by sedimentation equilibrium measurements on the solute in solutions of H₂O and D₂O or H₂O and D₂O¹⁸. This parallel determination of the concentration distributions at sedimentation equilibrium permits the evaluation of both \( \bar{\epsilon} \) and the molecular weight, \( M \), by solving the two simultaneous equations containing the two unknowns. While similar to the approach based on sedimentation velocity measurements in H₂O and D₂O solvents (11-17), the sedimentation equilibrium method has several advantages. Less material is required; knowledge of the viscosity of the solvent is unnecessary; the requirements imposed on the measurement of temperature are less stringent; complications due to pressure effects are substantially reduced; no ambiguity is introduced because of possible changes in the conformation of the macromolecules (with consequent differences in friction factors) in the various solvents; and, finally, equilibrium measurements can be made more readily with substances too small for accurate determination of sedimentation coefficients.
THEORY

For an ideal, two-component system the sedimentation equilibrium equation (Equation 1) takes the form

$$M(1 - \phi_0) = \frac{2RT}{\omega^2} \frac{d \ln c}{dr^2}$$

(1)

where $R$ is the gas constant, $T$ is the absolute temperature, $\omega$ is the angular velocity of the rotor in radians per sec, $c$ is the concentration of the redistributed solute, and $r$ is the distance from the axis of rotation. This equation, although valid for extremely dilute solutions, requires modification when the concentration is so high that terms accounting for nonideal behavior are not negligible (18–20). Similarly, when the solution contains more than two components an additional term must be included in Equation 1 to account for possible preferential interactions between the macromolecules and either H$_2$O or the third component (salt, sucrose, or urea, for example) (18–21). These preferential interactions may stem from attractive forces as in the selective binding of ions or, as suggested by Kauzmann (cited in Reference 22), from repulsion when H$_2$O appears preferentially "bound" due to steric exclusion of the third component. Such steric exclusion, when it does occur to any appreciable extent, is attributable to differences in the molar volumes of the different components in the mixed solvent (22, 23). In mixtures of H$_2$O and D$_2$O or H$_2$O and D$_2$O$^{18}$, however, such steric effects would seem to be negligible since the molar volumes of the different forms of water are in fact almost identical (24). Similarly, it would be expected that preferential binding of the different forms of water would not occur since the chemical properties of H$_2$O and D$_2$O, for example, are so similar. Thus H$_2$O-D$_2$O and H$_2$O-D$_2$O$^{18}$ solutions can be considered as one-component solvents.

Nearly all protein solutions, of course, are multicomponent systems since buffer ions are present in addition to the protein and the water. However, the salt concentration usually is so low for most buffers that the density of the solvent is only slightly greater than that of pure H$_2$O. As a consequence no significant hazards are introduced by neglecting the possible interactions between the protein and the H$_2$O (21). In effect, solutions of proteins in a buffer are tacitly considered two-component systems as if the buffer salts and H$_2$O formed a one-component solvent.

At high concentrations of the third (or fourth) component this assumption is inadmissible, and the inclusion of an additional term in Equation 1 is mandatory. Within the limitations outlined above, Equation 1 is an adequate representation of the concentration distribution for proteins in dilute buffer solutions made up of either H$_2$O or D$_2$O or mixtures of the two.

When Equation 1 is to be used for solutions containing either D$_2$O or D$_2$O$^{18}$, modifications are necessary to account for the changes in both $M$ and $\phi$ due to the replacement of exchangeable hydrogen atoms in the protein molecule by deuterium atoms (20). As a result of the deuterium exchange the molecular weight will attain the value, $kM$, where $k$ is the ratio of the molecular weight of the protein in the deuterated to that in the nondeuterated solvent. Since the change in the molar volume of the protein as a consequence of deuterium exchange can be considered negligible (16), the partial specific volume of the deuterated protein can be written as $\bar{\varepsilon}/k$. Consequently, for the macromolecules in D$_2$O, Equation 1 takes the form

$$kM \left(1 - \frac{\bar{\varepsilon}}{k} \rho_{\text{water}}\right) = \frac{2RT}{\omega^2} \frac{d \ln c}{dr^2} \rho_{\text{water}}$$

(2)

A similar equation applies for experiments in D$_2$O$^{18}$, where the experimental values for $\rho_{\text{D}_2\text{O}}$ and $(d \ln c/dr^2)_{\text{H}_2\text{O}}$ replace the analogous quantities for H$_2$O or D$_2$O solutions.

When parallel experiments on a given substance are performed at the same rotor speed and temperature Equations 1 and 2 can be combined and rearranged to give

$$\bar{\varepsilon} = \frac{k - \left[(d \ln c/dr^2)_{\text{D}_2\text{O}}/(d \ln c/dr^2)_{\text{H}_2\text{O}}\right] \rho_{\text{D}_2\text{O}} - \rho_{\text{H}_2\text{O}}}{[d \ln c/dr^2]_{\text{D}_2\text{O}}/(d \ln c/dr^2)_{\text{H}_2\text{O}}}$$

(3)

This shows that $\bar{\varepsilon}$ can be calculated readily from the concentration distributions in the two solvents coupled with knowledge of the densities of the respective solvents and the increase in the molecular weight resulting from deuterium exchange. With the value of $\bar{\varepsilon}$ calculated from Equation 3 the molecular weight is evaluated readily by substitution in Equation 1.

Since the term, $[(d \ln c/dr^2)_{\text{D}_2\text{O}}/(d \ln c/dr^2)_{\text{H}_2\text{O}}]$, appears in both the numerator and denominator of Equation 3 in the same form, errors in the measurement of the concentration distributions are not magnified in the final computation of $\bar{\varepsilon}$. For proteins, $(d \ln c/dr^2)_{\text{D}_2\text{O}}/(d \ln c/dr^2)_{\text{H}_2\text{O}}$ is about 0.8, and the corresponding quantity for D$_2$O$^{18}$ is approximately 0.5. Thus an experimental error of about 1% in the determination of the slopes of the plots of ln c with respect to $r^2$ leads to an error in $\bar{\varepsilon}$ of about 2% for experiments with D$_2$O, and less than 1% for analogous studies with D$_2$O$^{18}$.

The quantity, $k$, can be estimated with sufficient accuracy from knowledge of the number of exchangeable hydrogen atoms in the solute molecules (27). During the time required for the attainment of sedimentation equilibrium a large fraction of the potentially exchangeable hydrogen atoms would be replaced by deuterium. For proteins these exchangeable hydrogen atoms reside principally in the backbone polypeptide chain where there is 1 replaceable amide hydrogen atom per amino acid residue. A smaller number exist as nitrogen-, sulfur-, or oxygen-bound hydrogen atoms in the side chains of residues of amino acids such as lysine, cysteine, and serine. Direct measurements of the increase in the weight of bovine plasma albumin upon deuteration as well as independent experiments of the deuteration exchange in a variety of proteins led to a value of $k = 1.0155$ (16, 27). This in turn differs from that defined classically (1) when preferential interactions occur.
Partial Specific Volumes by Sedimentation in D$_2$O and D$_2$O$^{18}$

made on the basis of knowledge of the composition of the molecules. When the solvent contains D$_2$O (or D$_2$O$^{18}$) at concentrations significantly below 100% the value of $k$ is reduced proportionately. Thus for 1:1 mixtures of H$_2$O and D$_2$O, $k$ would be only about 1.008.

Fig. 1. Sedimentation equilibrium patterns for myoglobin in H$_2$O and D$_2$O$^{18}$ solutions. In the experiment two double-sector cells were used with multiplex operation of the photoelectric scanner (3). Cell 1 contained 4 mg of myoglobin in 0.1 ml of 0.1 M phosphate, pH 7.0, in H$_2$O. The traces representing this solution are at the left. Cell 2 contained 2.7 mg of myoglobin in the same buffer in 90% D$_2$O$^{18}$, and the corresponding traces are at the right. The D$_2$O$^{18}$ solution was prepared by gravimetric dilution of 0.1 ml of a myoglobin solution (containing 27 mg of myoglobin in 1.0 M phosphate at pH 6.7) with 0.9 ml of D$_2$O$^{18}$ (see Table I for details of the composition of D$_2$O$^{18}$). About 0.1 ml of fluorocarbon FC43 was added to each compartment of the double-sector cells in order to produce a transparent region at the cell bottom and thereby permit accurate measurements of the absorbance throughout the liquid columns. The traces were recorded, with light of wave length 405 m$\mu$ (28, 29); the traces at the top were obtained immediately after the rotor attained the equilibrium speed of 28,000 rpm. The time required for a scan of the image of the entire cell was 30 sec, and the trace amplitude was 400 (1,000 is the setting for maximum amplification). In the center are the traces after 16 hours of centrifugation. At the bottom are expanded and amplified traces showing only the regions of the cells corresponding to the myoglobin solutions. For these traces the scanning period was increased to 6 min so as to give an improved signal to noise ratio through the use of an electronic filter at the slower scanning rate. In these expanded traces the 1-mm column of the solution appeared as 20 cm on the final traces. In order to facilitate measurements of the concentration distribution the recorder deflection was amplified by adjustment of the helipot control (3) to 600 for Cell 1 and to 1,000 for Cell 2.

Fig. 2. Sedimentation equilibrium of $\alpha$-chymotrypsinogen in H$_2$O, D$_2$O, and D$_2$O$^{18}$ solutions. Two separate experiments were performed as described in the legend to Fig. 1. In the experiment represented by the plots at the left, the solution (containing 20 mg of $\alpha$-chymotrypsinogen in 0.1 ml of 0.1 M phosphate at pH 7 in H$_2$O) was placed in one compartment of a double-sector cell with solvent in the other compartment. The second cell contained the analogous solution of protein in D$_2$O in one compartment and solvent in H$_2$O in the other. For this experiment the speed was 36,000 rpm. In the second experiment (at 28,000 rpm), represented by the plots at the right, the solution in Cell 2 contained 90% D$_2$O$^{18}$, while that in Cell 1 contained the analogous solution and solvent in H$_2$O. Measurements were made from expanded traces of the type illustrated by the bottom patterns in Fig. 1, and the data were plotted as the logarithm of the recorder deflection against the square of the distance, $r$, from the axis of rotation. O, results in H$_2$O; $\bullet$, those in D$_2$O (or D$_2$O$^{18}$). The light used with the photoelectric scanner had a wave length of 280 m$\mu$. Since the D$_2$O$^{18}$ itself had a slight absorbance at this wave length due to an unidentified impurity, a corrected base line was established after the equilibrium patterns were recorded. This was accomplished by accelerating the rotor to 60,000 rpm and measuring the recorder deflection in the supernatant liquid (3).

RESULTS AND DISCUSSION

Fig. 1 shows typical recorder traces obtained with the photoelectric scanning absorption system in an experiment with myoglobin in H$_2$O in one double-sector cell and the same protein in D$_2$O$^{18}$ in the other. As seen in the patterns (experimental details are given in the legend to Fig. 1) the concentration change across the cell was much less for the cell containing myoglobin in D$_2$O$^{18}$. Representative plots of the data from two other experiments on $\alpha$-chymotrypsinogen are illustrated in Fig. 2. The plots of the logarithm of recorder deflection (which is proportional to concentration) with respect to the square of the distance from the axis of rotation were linear, as expected for homogeneous materials, with the slope in D$_2$O equal to 0.789 that for the protein in H$_2$O. For $\alpha$-chymotrypsinogen in D$_2$O$^{18}$ the slope was only 0.549 that for the protein in H$_2$O.

The values of $\bar{v}$ evaluated from the experiments illustrated by Figs. 1 and 2 are summarized in Table I along with other results. In addition, values of $\bar{v}$ obtained by standard methods with large quantities of protein are given for comparison. The results evaluated from the sedimentation equilibrium experiments with only microgram quantities of material are in excellent agreement with the values reported by other workers from studies based on density and concentration measurements. Even for small mole-
TABLE I
Measurement of $\bar{\eta}$ and $M$ by sedimentation equilibrium in $H_2O$ and $D_2O$ or in $H_2O$ and $D_2O^{18}$ solutions

All measurements were made with a Spinco model E ultracentrifuge equipped with a monochromator (28) and a split beam photometric scanner (29) as described previously (3). The experiments with myoglobin and ferredoxin the optical system was operated with light of wave length 280 nm. All other data were obtained with light of wave length 405 nm. The experiments on bovine plasma albumin and myoglobin in $H_2O$ and $D_2O$ solutions were performed with a multichannel double-compartment cell (30), while the remainder were conducted with two double-sector cells in the same ultracentrifuge experiment through the use of the multiplex attachment on the scanning system (3). All sedimentation equilibrium experiments were conducted at 20° with 3-mm columns of liquid. The duration of the experiments was about 16 hours, and the attainment of equilibrium was checked by superposition of the traces taken at intervals of several hours. For each experiment the plot of the logarithm of the recorder deflection with respect to the square of the distance from the axis of rotation was linear, and the slope was evaluated from the least squares fit of the data. $D_2O$ (at least 99.5%) was obtained from Matheson, Coleman, and Bell, Division of Matheson Company, Inc., Norwood (Cincinnati), Ohio. D$_2$O solvents (including buffer salts) were obtained from the International Critical Tables. D$_2$O solutions containing $D_2O^{18}$ were determined pycnometrically. For solvents containing $D_2O^{18}$, the densities were calculated from the expression of Steckel and Szapiro (24)

$$\bar{\eta}/\eta_{\text{max}} = 1 - \frac{1}{10m} \left(1 + \frac{482.502}{\tau + 77.861}\right)$$

where $\tau = t - t_{\text{max}}$, $t$ is the temperature for which the density, $\rho$, is desired, and $t_{\text{max}}$ is the temperature at which $D_2O^{18}$ has its maximum density ($\rho_{\text{max}}$). For $D_2O^{18}$, $\rho_{\text{max}} = 1.21691 \pm 0.00008$ at $t_{\text{max}} = 11.46 \pm 0.03$°. All solutions in $D_2O$ and $D_2O^{18}$ were prepared by gravimetric dilution of concentrated protein-buffer-$H_2O$ solutions. The resulting solutions contained about 90% $D_2O$ or $D_2O^{18}$. The stock solution of $D_2O^{18}$ contained 97.99% $D_2O$, 0.68% $O_2$, and 99.30% $D$.

<table>
<thead>
<tr>
<th>Solvent pair and solute</th>
<th>Partial specific volume</th>
<th>Molecular weight</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Sedimentation equilibrium</td>
<td>Literature</td>
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<tr>
<td>$H_2O-D_2O$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenosine$^{a}$</td>
<td>0.62</td>
<td>0.63 (31)</td>
</tr>
<tr>
<td>Ferredoxin$^{c}$</td>
<td>0.61</td>
<td>0.63 (32)</td>
</tr>
<tr>
<td>Ribonuclease$^{c}$</td>
<td>0.70</td>
<td>0.69 (33)</td>
</tr>
<tr>
<td>Myoglobin$^{a}$</td>
<td>0.74</td>
<td>0.74 (35)</td>
</tr>
<tr>
<td>$\alpha$-Chymotrypsinogen$^{a}$</td>
<td>0.72</td>
<td>0.72 (37)</td>
</tr>
<tr>
<td>Bovine plasma albumin$^{b}$</td>
<td>0.73</td>
<td>0.73 (6)</td>
</tr>
<tr>
<td>$H_2O-D_2O^{18}$</td>
<td></td>
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</tr>
<tr>
<td>Myoglobin$^{c}$</td>
<td>0.74$^c$</td>
<td>0.74 (35)</td>
</tr>
<tr>
<td>$\alpha$-Chymotrypsinogen$^{a}$</td>
<td>0.71$^c$</td>
<td>0.72 (37)</td>
</tr>
</tbody>
</table>

$^a$ Obtained from Nutritional Biochemicals. The solvent was 0.2 M NaCl and the speed was 60,000 rpm. $^b$ Clostridial ferredoxin was kindly provided by J. C. Rabitz and R. Malkin. The solvent was 0.1 M NaCl—0.01 M Tris at pH 7.4, and the speed was 60,000 rpm. $^c$ Obtained from Worthington Biochemical Corporation, Freehold, New Jersey, Lot RAS 6018, chromatographically purified. The solvent was 0.1 M NaCl-0.01 M phosphate at pH 6.4, and the speed was 52,000 rpm. $^d$ Myoglobin (spem whale) was obtained from Mann, Lot K 1387. The solvent was 0.1 M phosphate at pH 7.0, and the speed was 42,040 rpm. $^e$ The value of $k$ (1.0149) was based on the protein part of the molecule. $^f$ Bovine pancreatic $\alpha$-chymotrypsinogen, A grade, Lot 49936, was obtained from Calbiochem. The solvent was 0.1 M phosphate at pH 7.0, and the speed was 30,000 rpm.

Table I shows also that reliable molecular weights as well as partial specific volumes can be evaluated from simultaneous sedimentation equilibrium measurements in $H_2O$ and $D_2O$ (or $D_2O^{18}$). The discrepancies which appear in Table I, as in the case of adenosine, for example, are attributable to the imprecision in $\bar{\eta}$. If a value of 0.63 ml per g is used in Equation 1 along with the measured value of $d$ in $c/dr^2$, the calculated molecular weight is 264, in excellent agreement with the known value. It should be noted that the sedimentation equilibrium method actually provides a measure of $(1 - \bar{\eta})$ and that $\bar{\eta}$ and the results in terms of $\bar{\eta}$ leads to an apparent reduction of the experimental error. Although the experiments reported here were performed with the photoelectric scanning absorption optical system the method is not restricted to that optical technique. Schlieren or interference optics will suffice as well.4 With the Rayleigh interference system the concentration distribution can be measured more precisely, thereby leading to increased accuracy in the final determination of $\bar{\eta}$ (and of $M$). When pure $H_2O^{18}$ becomes readily available the present (slight) ambiguity resulting from uncertainty in the extent of deuterium exchange would be eliminated. If $H_2O^{18}$ were to be used routinely, an increase in the precision in measuring the concentration distributions would be desirable. Since the method presented here is based, in principle, on the determination of the difference in the redistribution of the solute molecules, further progress might be achievable by sub-

4 In ultracentrifuge experiments on $H_2O-D_2O$ mixtures at 60,000 rpm virtually no refractive index gradient has been observed. Analogous experiments on solutions of $H_2O$ and $D_2O$ have not been performed, but no significant redistribution of the heavier molecular species is expected at the centrifugal fields now available since the molecular weight and partial specific volume of $D_2O^{18}$ are so close to those of $H_2O$. 

TABLE I—Continued
tracting directly the concentration curves for the two samples (in H₂O and in D₂O₁₈, for example) through the use of the split beam scanning system (30). This possibility, as yet, has not been explored.

Measurements of \( \delta \) can be made with ease at 0° by the sedimentation equilibrium method whereas such determinations by standard experimental techniques are fraught with difficulty. The method described and illustrated here not only has the advantage of providing reliable results with only micrograms of protein and without requiring accurate dry weight concentrations, but also, under certain circumstances, can yield satisfactory results even with impure preparations. If the substance of interest has unique spectral properties its redistribution at sedimentation equilibrium can be measured with the scanning system despite the presence of contaminants in the preparation (30). Even in the presence of small amounts of impurities having similar absorption spectra but markedly different molecular weights, \( \delta \) for the principal component can be determined by judicious selection of the rotor speed for the sedimentation equilibrium measurements (3, 30). Under ideal circumstances the method can yield values of \( \delta \) accurate to about ±0.003 ml per g, but in terms of accuracy the sedimentation equilibrium technique cannot rival the standard methods. In addition severe limitations are imposed on the sedimentation method with associating-dissociating systems.

In effect the method described here is analogous to sedimentation methods (3, 11–17, 29, 40–48) which provide \( B \) as the reciprocal of the partial specific volume of proteins is about 1.35 ml, corresponding to a partial specific volume for the complex. By way of example, the density of phosphate buffer corresponding to zero redistribution of the protein has been found to be only 1.20 g per ml, the value of the partial specific volume corresponding to zero redistribution can only be obtained by direct experimentation; instead it is evaluated indirectly. Clearly the accuracy of the method could be enhanced if solutions of greater density were available. But these cannot be achieved by adding a dense, third component such as inorganic salts, urea, or guanidine hydrochloride without incurring the risk of preferential interactions. When such interaction occurs the value of the partial specific volume corresponds to that of the complex between the principal solute (protein) and the preferentially bound component (3). With myoglobin, for example, the density of phosphate buffer corresponding to zero redistribution has been found to be only 1.20 g per ml, corresponding to a partial specific volume for the complex of 0.83 ml per g. This is to be contrasted to the value of 5.074 ml per g, obtained in D₂Ο₁₈. A combination of the data from the two types of experiments provides the information needed to assess the extent of the preferential interactions (3, 18–22, 44). If water containing exclusively tritium and O¹⁸ (T₂O₁₈) were available and could be used for ultracentrifuge studies despite its instability, the accuracy in determining \( \delta \) would be enhanced markedly since the density of such solutions would be greater than 1.3 g per ml. As long as the molar volumes and chemical properties of T₂O₁₈ and H₂O were similar there would be little risk of preferential interactions.

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