Contribution of the Excluded Volume of Bovine Serum Albumin for Solute Molecules to the Apparent Nonsolvent Water

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ABSTRACT  Addition of a macromolecule to a solution will give rise to a large excluded volume for the centers of the solute molecules. This will cause an apparent increase in solute concentration which is of the same order of magnitude as that associated with the nonsolvent volumes reported in the literature. A critical examination of one of the procedures used for the determination of nonsolvent water—the vapor pressure method of Hill—is given, and it is concluded that, with the use of this method, it is impossible to detect any significant nonsolvent water surrounding bovine albumin for either sugars or polyols. Generally, data reported in the literature for the nonsolvent water of proteins or other macromolecules will be too high unless they are corrected for the excluded volume.

INTRODUCTION

Several methods for the determination of the nonsolvent water surrounding proteins are in use. They consist essentially of determining the change in concentration of a solute caused by addition of a protein to the solute solution. This concentration can be determined directly in the ultrafiltrate of the protein solution (Greenberg and Greenberg [1]), or in the dialysate (Gary-Bobo [2]), or indirectly by measuring the change in freezing point (Sunderman [3]), or the change in vapor pressure (Hill [4]). Also studies of the distribution of neutral solutes between cells and medium can be used (Miller [5], MacLeod and Ponder [6], Troshin [7], Gary-Bobo [2], and Cook [8]). Determinations of the nonsolvent water in protein crystals have been carried out by determining the concentration of a solute in crystal water and comparing this concentration with the concentration in the mother liquid (Perutz [9], McMeekin et al. [10], Drabkin [11]). Values of nonsolvent volume found via these different methods generally vary from about 0.4 ml of water.
per g of protein to virtually zero. This may be partly due to differences in methods or experimental conditions. It has been found by Roepke and Baldes (12) that the relative increases in osmotic value determined by means of the vapor pressure method of Hill depend upon the solute used. This has been confirmed by Gary-Bobo (2) in a study of the distribution of solutes over red cells and medium. The differences in nonsolvent volume observed with varying solutes may indicate that the hydration layer around the protein permits the entrance of solute molecules to varying extents depending on the nature of the solute.

Schachman and Lauffer (13) have pointed out that the apparent hydration of tobacco mosaic virus depends upon the size of the solute present when the hydration is determined. This is due to the fact that the centers of the solute molecules are excluded to different extents from the surface of the virus. When this exclusion is larger than the exclusion of the solvent molecules, an apparent increase in concentration of the solute will result. Therefore, the amount of nonsolvent water calculated will be too large, and must be corrected for the contribution of the excluded volume. This correction depends upon the size of the solute molecule, and also upon the surface area of the macromolecule or particle involved. A dependence upon solute size of the exclusion for solutes from the surface of macromolecules has been observed by Ogston and Phelps (14) for hyaluronic acid, and by Laurent (15) for both hyaluronic acid and dextran.

We have now calculated the expected excluded volume of a number of polar solutes in the presence of bovine serum albumin. In addition the vapor pressure method of Hill has been applied in order to determine the increase of osmotic value occurring upon the addition of protein to solute solutions. This increase is corrected for the calculated contribution of the exclusion effect to the osmotic value. It will be shown that, for polyols and carbohydrates, the net increase in osmotic value is virtually zero.

**METHODS**

Osmotic values of solutions were determined with a Mechrolab vapor pressure osmometer (Hewlett-Packard, Avondale, Pa.) at 37°C. The readings were made 4 min after applying the samples to the thermistors. A linear relationship exists between the amount of resistance change needed to rebalance the Wheatstone bridge and the osmotic values of NaCl (see Ts'o, Melvin, and Olson [16]). This has been confirmed by us. Osmotic coefficients given by Robinson and Stokes (17) are used for the calculation of the osmotic values of NaCl. These osmotic coefficients are given for 25°C, but the coefficients do not change much in the temperature range involved. The mean deviation of determinations of osmotic values was 2.8 instrumental divisions, corresponding to 0.4 milliosmolar. This is in accordance with the experience of Ts'o et al. (16).

The concentrations of solutes in mixtures of solute and water, expressed in milli-
molal \((C_0)\), are calculated by dividing the osmotic value (milliosmolal) by the osmotic coefficient. The volume of solute in 1 ml of solution is calculated according to equation (1).

\[
v_s = C_0(1 - v_s)M_s/10^6 d_s = C_0M_s/(10^6 d_s + C_0M_s)
\]

\((M_s)\) is the molecular weight of the solute, and \((d_s)\) is the density of the solute.

Determinations of intrinsic viscosities \([\eta]\) expressed in milliliters per gram were made with a glass capillary viscometer at 25°C. The figures refer to the weight of solutes without crystal water. Determinations of densities were made with pycnometers at about 22°C. These values also refer to dry solutes without crystal water. Determinations of dry weight were made by heating the compounds to constant weight at 105°C, unless the compounds were volatile. In this case, we used a desiccator filled with \(P_2O_5\) at room temperature.

The protein used was bovine serum albumin fraction V from Sigma Chemical Co. (St. Louis, Mo.) and in a few cases bovine serum albumin from Armour Pharmaceutical Co. (Kankakee, Ill.). The pH of a solution of 200 mg bovine albumin in 1 ml of water was 5.10 ± 0.05 at about 22°C. The density \((d_m)\) was 1.36.

**THEORY**

We are dealing with a three-component system: water \((w)\), solute \((s)\), and macromolecule \((m)\). As already pointed out by Schachman and Lauffer (13) the presence of macromolecules with a large surface area will give rise to an excluded volume for the centers of solute molecules. We will calculate the magnitude of this volume for the macromolecule bovine serum albumin. It is assumed that the mean distance from the centers of the solute molecules to the protein surface is equal to the radius \((r_s)\) of the equivalent sphere of unhydrated solute:

\[
r_s = (3M_s/4\pi N_A d_s)^{1/3} \text{cm}
\]

where \(N_A\) is Avogadro's number. For \(r_w\) we have used both the value 1.45 Å, the mean distance between oxygen atoms in the Danford-Levy model (see Berendsen [18]) and the value 1.93 Å calculated via equation (2).

Riddiford and Jennings (19) consider bovine serum albumin to be a prolate spheroid with semiaxes of 57.0 Å and 18.4 Å. The total excluded volume for a solution containing \(n\) macromolecules will be given by equation (3)

\[
v_{w.o} = n(4/3)\pi[(a + r_w)(b + r_s^2) - (a + r_w)(b + r_w)^2]
\]

\[= 3.766 \times 10^{-5}g(2574 + 95.25r_s + r_s^2)(r_s - 1.45)\] \(3a\)

or

\[3.766 \times 10^{-5}g(2619 + 95.73r_s + r_s^2)(r_s - 1.93)\] \(3b\)
(3a) and (3b) refer to \( r_w = 1.45 \times 10^{-8} \text{ cm} \) and \( r_w = 1.93 \times 10^{-8} \text{ cm} \), respectively.

\( v_{e,o} \) is given in milliliters when expressing \( a, b, r, \) and \( r_w \) in centimeters; \( n \) is \( g \times N_A/M = 8.99 \times 10^{18} \text{ g} \); \( N_A \) is Avogadro's number; \( M \) is 67,000, the molecular weight of bovine serum albumin; and \( g \) is the weight of protein in grams added to the solution of \( s \) in \( w \), and corrected for the moisture content of the protein. The excluded volume has to be corrected because part of the volume is occupied by other proteins and because part of the volume is taken twice because of the overlapping of the excluded volume of two molecules. These corrections are proportional to the volume fraction of the macromolecules \( (v_m/v_t) \) and to half the volume fraction occupied by the excluded volume \( (v_e/v_t) \). \( v_m \), \( v_e \), and \( v_t \) are the volumes occupied by the protein, the corrected excluded volume, and the total volume of the solution, respectively. The corrected excluded volume is given by:

\[
v_e = v_{e,o}(1 - v_m/v_t - 0.5v_e/v_t) = v_{e,o}(v_t - v_m)/(v_t + 0.5v_{e,o}) \tag{4}
\]

\( v_m \) is equal to the product of the dry weight of the protein added and the reciprocal value of the density of the protein. The space available for the solute in the presence of the macromolecule will decrease from \( (v_t - v_m) \) to \( (v_t - v_m - v_e) \) because of the excluded volume. It is now possible to calculate the apparent nonsolvent volume \( (ANSW) \) due to the excluded volume and to express this in grams per g of protein by dividing \( v_e \) by the dry weight of the protein \( (g) \):

\[
ANSW = v_e/g \tag{5}
\]

Experimental values of apparent nonsolvent volume are calculated from the decrease in water available for the solute; i.e., from the increase in solute concentration. It is assumed that the ratio of solute concentrations is equal to the ratio of contributions of the solute to the total osmotic value. This will be true when the osmotic coefficients do not change on adding the protein. The ratios are given by:

\[
C_s/(C_s + dC_s) = P_s/(P_s + dP_s) = (v_t - v_m - v_e)/(v_t - v_m) \tag{6}
\]

\( dC_s \) and \( dP_s \) are the increases in concentration and in osmotic contribution of the solute, respectively.

The value for the experimental apparent nonsolvent volume calculated from the increase in osmotic value is given by:

\[
ANSW_{exp} = v_e/g = dP_s(v_t - v_m)/(P_s + dP_s)g \approx dP_s/(P_s + dP_s)g \tag{7}
\]

The last approximation is allowed in the special case when a certain amount
of protein is added to 1 ml of solution. Then $v_i - v_m$ is approximately 1 ml provided that the moisture content of the added protein is not too high. In that case (4) approximates to:

$$v = v_{eq} / (1 + g d_m^{-1} + 0.5v_{eq})$$  \hspace{1cm} (8)

**RESULTS**

We have determined the osmotic coefficients of the solutes which are necessary for the calculation of $C_s$ and of $v_s$, the volume of the solute. The osmotic coefficients are calculated by means of the experimental relation:

$$f_s = 1 + aC_s$$  \hspace{1cm} (9)

Table I includes the values of $a$, the values for the radii of the solutes ($r_s$), the densities of the solutes in the solution ($d_s$), the intrinsic viscosities [$\eta$],

**TABLE I**

**PROPERTIES OF SOLUTE MOLECULES USED IN THE EXPERIMENTS**

| Solute       | $a$   | $r_s$ | $d_s$ | [$\eta$] | [$\eta$]/2.29 |
|--------------|-------|-------|-------|----------|--------------|
| Glucose      | 3.55  | 0.63  | 2.25  |          |              |
| Galactose    | 3.52  | 0.61  | 2.20  |          |              |
| Sorbose      | 3.52  | 0.61  | 2.27  |          |              |
| Sucrose      | 0.00009 (17) | 4.38  | 0.62  | 2.24     |              |
| Maltose      | 0.00009* | 4.38  | 0.62  | 2.35     |              |
| Melibiose    | 0.00009* | 4.38  | 0.62  | 2.47     |              |
| Gellubiose   | 0.00009* | 4.38  | 0.62  | 2.53     |              |
| Raffinose    | 0.00016† | 5.03  | 0.64  | 2.30     |              |
| Stachyose    | 0.00020§ | 5.49  | 0.63  |          |              |
| Glycol       | 2.78  | 0.87  | 2.23  |          |              |
| Glycerol     | 3.08  | 0.80  | 1.97  |          |              |
| Mannitol     | 3.61  | 0.65  | 2.39  |          |              |
| Sorbitol     | 3.61  | 0.65  | 2.38  |          |              |
| Inositol     | 3.42  | 0.56  | 2.23  |          |              |
| PEG 200      | ±0.00006† | 4.07  | 0.85  | 3.15     | 1.38         |
| PEG 300      | 0.00025| 4.69  | 0.87  | 3.61     | 1.58         |
| PEG 400      | 0.00045| 5.13  | 0.85  | 4.00     | 1.75         |
| PEG 600      | 0.00078† | 5.84  | 0.84  | 4.73     | 2.07         |
| PEG 1000     | 0.0025‡ | 6.93  | 0.84  | 5.91     | 2.58         |

$a$ is the coefficient of $C_s$ in equation 9.

* $a$ is assumed to be equal to the value for sucrose.

† Determined experimentally by vapor pressure osmometry.

§ Estimated by extrapolating a plot of $a$ against the molecular weight of sucrose and raffinose.

‖ Estimated by interpolating the plot of $a$ against the molecular weight of the other PEG molecules. $r_s$ is the radius of the solute moleule calculated according to equation (1); $d_s$ is the density of the solute; [$\eta_s$] is the intrinsic viscosity of the solute. [$\eta_s$]/2.29 is the ratio of the intrinsic viscosities of the PEG solutes divided by the mean viscosity of the other molecules.
and the ratio of the intrinsic viscosities of the polyethylene glycol solutes and the mean value of the viscosities of the other molecules.

Table II summarizes the results of our experiments, in which we determined the differences \((dP)\) in osmotic values of mixtures of solute, protein, and water \((P_{m+w})\), and the sum of the osmotic values of protein and water \((P_m)\) and of solute and water \((P_s)\).

The procedure followed consists essentially in determining the osmotic values of 200 mg of protein added to 1 ml of water, the osmotic value of a solution of nonelectrolyte, and the osmotic value of a mixture of 200 mg of protein added to 1 ml of solution. In order to obtain osmotic values for the solute solution and the protein solution which may be compared with the

| Solute  | g   | \(P_m\) | \(\pm SE\) | \(C_s\) | \(v_s\) | \(P_s\) | \(\pm SE\) | \(P_{m+s}\) | \(\pm SE\) | \(dP_s\) | \(\pm SE\) |
|---------|-----|---------|------------|--------|--------|---------|------------|------------|--------|----------|--------|
| Glucose | 0.192 | 175 | 1.4 | 5 | 112 | 0.012 | 775 | 6 | 2 | 965 | 3 | 2 | 15 | 7 |
|         | 0.192 | 183 | 1.4 | 5 | 202 | 0.023 | 1372 | 0 | 2 | 1560 | 1 | 34 | 2 |
| Galactose | 0.188 | 194 | 1.2 | 9 | 203 | 0.023 | 1391 | 1 | 5 | 1620 | 4 | 5 | 35 | 4 |
| Sorbose  | 0.188 | 196 | 1.2 | 9 | 153 | 0.031 | 1905 | 2 | 3 | 1313 | 1 | 3 | 52 | 3 |
| Sucrose  | 0.188 | 196 | 1.2 | 9 | 137 | 0.028 | 955 | 1 | 2 | 1193 | 12 | 2 | 42 | 12 |
| Maltose  | 0.192 | 165 | 1.5 | 3 | 483 | 0.093 | 3350 | 37 | 2 | 3635 | 1 | 120 | 37 |
|         | 0.192 | 200 | 1.7 | 6 | 91 | 0.019 | 630 | 2 | 3 | 865 | 7 | 3 | 35 | 7 |
| Melibiose | 0.192 | 177 | 1.4 | 5 | 113 | 0.024 | 787 | 2 | 2 | 1021 | 2 | 37 | 3 |
|         | 0.188 | 201 | 1.7 | 6 | 113 | 0.024 | 784 | 2 | 2 | 1027 | 2 | 42 | 3 |
| Celllobiose | 0.192 | 178 | 1.4 | 5 | 135 | 0.027 | 942 | 1 | 1 | 1169 | 1 | 49 | 2 |
| Raffinose | 0.188 | 195 | 1.2 | 9 | 79 | 0.024 | 550 | 1 | 3 | 785 | 4 | 40 | 4 |
| Stachyose | 0.188 | 200 | 1.7 | 6 | 47 | 0.019 | 328 | 2 | 2 | 354 | 2 | 9 | 5 |
| Glycol   | 0.188 | 194 | 1.2 | 9 | 345 | 0.019 | 2356 | 0 | 2 | 2612 | 3 | 3 | 52 | 3 |
| Glycerol | 0.188 | 206 | 1.7 | 6 | 481 | 0.032 | 3283 | 4 | 2 | 3594 | 0 | 2 | 90 | 4 |
| Mannitol | 0.192 | 175 | 1.4 | 5 | 102 | 0.012 | 701 | 2 | 2 | 898 | 4 | 2 | 22 | 5 |
| Sorbitol | 0.192 | 177 | 1.4 | 5 | 192 | 0.022 | 1322 | 1 | 1 | 1557 | 2 | 58 | 8 |
| Inositol | 0.188 | 201 | 1.7 | 6 | 266 | 0.026 | 1824 | 2 | 3 | 2102 | 1 | 2 | 77 | 3 |
| PEG 200 | 0.188 | 204 | 1.7 | 6 | 212 | 0.026 | 1474 | 2 | 2 | 1815 | 11 | 2 | 137 | 11 |
| 300     | 0.188 | 203 | 1.7 | 6 | 123 | 0.022 | 872 | 2 | 2 | 1186 | 0 | 2 | 111 | 3 |
| 400     | 0.188 | 204 | 1.7 | 6 | 113 | 0.029 | 815 | 0 | 2 | 1186 | 2 | 167 | 2 |
| 600     | 0.188 | 202 | 1.7 | 6 | 55 | 0.028 | 398 | 2 | 2 | 710 | 11 | 2 | 110 | 11 |
| 1000    | 0.188 | 193 | 1.4 | 5 | 219 | 0.104 | 1668 | 3 | 2 | 2237 | 7 | 2 | 406 | 8 |

\(g\) is the number of grams of dry protein corresponding to 0.2 g of wet protein. \(P_m\), \(P_s\), and \(P_{m+s}\) are the osmotic values, expressed in instrument units (1 milliosmolal = 6.95 units), of the protein in water, of the solute in water, and of mixtures of protein, water, and solute. \(C_s\) is the millimolar solute concentration. \(dP_s\) is \(P_{m+s} - P_m - P_s\). \(v_s\) is the volume of the solute in the solution. \(n\) is standard error, \(n\) is number of determinations.
osmotic value of the solution of protein and solute together, some corrections have to be applied. The protein contains some water (x ml per 200 mg) and this leads to a small dilution of the solute. On the other hand, the water content of 1 ml of solution is less than 1 ml, being equal to 1 - v. The osmotic value found for the solute is multiplied by (1 - v)/(1 - v + x),

\[ \text{ANSW}_{\text{exp}} = \text{ANSW} \times \frac{1 - v}{1 - v + x} \]

### Table III

Comparison of experimentally found apparent nonsolvent values with amounts of apparent nonsolvent water theoretically expected due to the excluded volume effect

| Solute   | \( \text{ANSW}_{\text{exp}} \) | \( \text{ANSW}_{0,0} \) | \( \text{ANSW}_{1,8} \) | \( \text{ANSW}_{\text{exp}} / \text{ANSW}_{0,0} \) | \( \text{ANSW}_{\text{exp}} / \text{ANSW}_{1,8} \) |
|----------|-------------------------------|--------------------------|------------------------|-----------------------------------------------|-----------------------------------------------|
| Glucose  | 0.099                         | 0.199                    | 0.157                  | 0.50                                          | 0.63                                          |
|          | 0.176                         | 0.199                    | 0.157                  | 0.68                                          | 1.12                                          |
| Galactose| 0.136                         | 0.199                    | 0.156                  | 0.68                                          | 0.87                                          |
| Sorbose  | 0.131                         | 0.197                    | 0.155                  | 0.66                                          | 0.85                                          |
| Sucrose  | 0.248                         | 0.285                    | 0.243                  | 0.87                                          | 1.02                                          |
| Maltose  | 0.180                         | 0.294                    | 0.242                  | 0.63                                          | 0.74                                          |
| Melibiose| 0.352                         | 0.284                    | 0.242                  | 1.24                                          | 1.45                                          |
| Cellobiose| 0.028                         | 0.284                    | 0.242                  | 0.91                                          | 1.06                                          |
| Raffinose| 0.361                         | 0.354                    | 0.313                  | 1.02                                          | 1.15                                          |
| Stachyose| 0.142                         | 0.404                    | 0.363                  | 0.35                                          | 0.39                                          |
| Glycol   | 0.114                         | 0.124                    | 0.081                  | 0.92                                          | 1.41                                          |
| Glycerol | 0.141                         | 0.134                    | 0.111                  | 0.92                                          | 1.28                                          |
| Mannitol | 0.139                         | 0.205                    | 0.163                  | 0.77                                          | 0.97                                          |
| Sorbitol | 0.219                         | 0.205                    | 0.163                  | 1.07                                          | 1.34                                          |
| Inositol | 0.216                         | 0.190                    | 0.145                  | 1.13                                          | 1.49                                          |
| PEG 200  | 0.452                         | 0.233                    | 0.211                  | 1.79                                          | 2.15                                          |
| 300      | 0.601                         | 0.318                    | 0.276                  | 1.89                                          | 2.18                                          |
| 400      | 0.905                         | 0.365                    | 0.324                  | 2.48                                          | 2.80                                          |
| 600      | 1.152                         | 0.443                    | 0.402                  | 2.60                                          | 2.87                                          |
| 1000     | 1.453                         | 0.566                    | 0.526                  | 2.57                                          | 2.76                                          |

\( \text{ANSW} \) means apparent nonsolvent water expressed in g per g of protein and calculated according to equation (5). \( \text{exp} \) denotes experimental values obtained when using the experimentally found values of \( dP_e \) and \( P_t \) for the calculation of \( \text{ANSW} \). \( \text{ANSW}_{0,0} \) and \( \text{ANSW}_{1,8} \) are the apparent nonsolvent water values calculated from theoretical values of \( v \) (equation 4) for \( r_h \) is 1.45 Å and \( r_p \) is 1.93 Å, respectively. Osmotic values, concentrations of solute, and amounts of protein are given in Table II.

giving \( P_a \); and the osmotic value of the protein in 1 ml of water is divided by \( (1 - v) \), giving \( P_a \). The latter correction is based upon the fact that an almost linear relation exists between the osmotic value of the protein solution and the amount of protein added to 1 ml of water, up to a concentration of 0.3 g of protein per ml.
Calculation of the contribution of the protein at a concentration of 200 mg per ml to the osmotic value and the use of the data of Scatchard et al. (20) on the osmotic coefficient of bovine albumin reveal that about 80% (differing somewhat from batch to batch) of the osmotic value is to be ascribed to other particles than the protein. This value corresponds to about 20 milliosmotic and presumably caused by salts present in the protein samples.

Apparent non-solvent values calculated from the values of \( dP \) and \( P \) according to equation (7) are given in Table III and are compared with theoretical values of the apparent non-solvent value calculated from the excluded volume (see equation [5]). It appears that the differences between experimental apparent non-solvent values and the calculated values do not differ much. The apparent non-solvent value increases with increasing molecular volume for the series of the saccharides. The mean values for the monosaccharides, the disaccharides, and the trisaccharides are 0.136, 0.258, and 0.361, respectively. The value for stachyose, however, is much smaller than expected. An increase in apparent non-solvent water with molecular weight is also seen in the series of the polyols and of the PEG (polyethylene glycol) solutes. Comparison of experimental values for apparent non-solvent values and those derived from calculated excluded volumes shows that both values are about the same in the case of sugars and polyols. The values calculated for the PEG solutes, however, are much smaller than those observed. This may be partly due to the fact that the effective radii of the PEG solutes are larger than those calculated from their molar volume.

Finally our results show that the apparent non-solvent values do not differ much within the range of water radii representing two extreme values.

**DISCUSSION**

It appears from the calculations of the apparent non-solvent volume (ANSW in Table III) that these values are rather high, and, in several cases, of the same order of magnitude as the values reported in the literature for non-solvent volumes (see the Introduction). These non-solvent values must be corrected for the contribution of the exclusion effect. The amounts of non-solvent water surrounding hemoglobin, determined using ethylene glycol, glycerol, and mannitol (2), do not differ by much from the corresponding values for the excluded volumes calculated for these compounds in the case of bovine albumin. This suggests that the hydration water of hemoglobin is almost completely accessible to these compounds. The values of the non-solvent water of hemoglobin calculated by Roepke and Baldes (12) from results of vapor pressure studies with glycerol, glucose, and sucrose are also of the same order of magnitude as those calculated for the excluded volume around bovine albumin. With gelatin some higher values were found using glycerol and mannitol as solutes. Since the nonglobular protein, gelatin,
has a much larger surface than globular proteins, the excluded volume will be larger and will probably account for the higher values of nonsolvent water calculated.

Our own data may only give an indication of the presence of nonsolvent water in the case of the PEG solutes. The increase in osmotic values of polyol or sugar solutions found after addition of bovine albumin can almost quantitatively be accounted for by the "solute exclusion effect." This means that the vapor pressure studies conducted with these strongly polar solutes do not give any indication for the existence of a nonsolvent water layer around bovine albumin. However, we are aware that the osmotic values of protein solutions and mixtures of protein, solute, and water depend upon several factors. The possibility cannot be excluded, for example, that the effect of true nonsolvent water may be compensated for by other effects, such as the adsorption of solutes to specific groups at the protein surface. It is also possible that the increase in osmotic value observed on mixing protein and solution is due to an increase in the activity of the salts present in the protein solutions. It appeared, however, that the addition of either a polysaccharide or polyethylene glycol to a salt solution affected the osmotic value to only a small extent. The use of 1.8% w/v of raffinose gave rise to a decrease of 1% in the osmotic value of 25 mmolal NaCl, and 7% w/v of polyethylene glycol 6000 only raised the osmotic value of 100 mmolal NaCl by 14%. These percentage changes in osmotic value are much smaller than the increases calculated for mixtures of solute, protein, and water even when the computations are made using the entire osmotic value of the protein solution as the base line.

The possibility that the results were affected by the presence of salts may not be overlooked. It may be that the results in a salt-free, though very unphysiological, medium may be quite different. In addition, our experiments were carried out at rather high concentrations of nonelectrolytes. This may also affect the experimental results in some manner.

The calculated increase in the apparent nonsolvent value is much smaller than that observed experimentally in the case of the polyethylene glycol solutes. It may not be justified, however, to calculate the radii of the polyethylene glycol molecules from their molar volume. The intrinsic viscosities of these molecules are much larger than the intrinsic viscosities found for the other solutes, indicating that the effective radii are larger than those calculated. The ratio of the observed increase in apparent nonsolvent value to the calculated one increases with the molecular weight of the polyethylene glycol molecules. This is also the case with the ratio of the intrinsic viscosities of these molecules and the mean intrinsic viscosity of the other solutes. This means that at least a considerable part of the increase in apparent nonsolvent value is due to the exclusion of the polyethylene glycol molecules from the
protein surface. Whether or not some real nonsolvent water is present cannot be decided from our present experimental results.

It has been emphasized by Gary-Bobo (2) that a number of variables must be taken into account when dealing with the question of nonsolvent water. Our study shows, in addition, that the effect of the exclusion of solutes cannot be neglected. In some cases it can even account for the total amount of apparent nonsolvent water calculated. Furthermore, estimates of nonsolvent water deduced from the anomalous osmotic behavior of red cells (see for example references 21 and 22) are probably too high and should not only be corrected for the effect of the mutual exclusion of the protein molecules themselves (see Dick [23]), but also for the contribution due to exclusion of solutes from the protein surface.

A possible consequence of the exclusion of solutes from the surface of macromolecules has already been discussed by Law and Phelps (24) in connection with determinations of the free space of cells. It is to be expected that larger solutes will give rise to smaller free spaces than small compounds do. This has been confirmed experimentally by these authors. Maizels and Remington (25) also found much smaller values for the intercellular space of red blood cells when they used proteins as solute than when they used inulin or lactose.

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