INTERPRETATION OF OSMOTIC PRESSURE IN SOLUTIONS OF ONE AND TWO NONDIFFUSIBLE COMPONENTS

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ABSTRACT Osmotic pressure data from aqueous solutions of nondiffusible serum albumin (BSA), chondroitin sulfate (CHS), and dextran T110 (D110), taken singly and in binary combinations, were interpreted in terms of excluded volume. The principal solvent was phosphate-buffered saline, pH 7.2, at 23°C. Osmotic pressures were measured with a membrane osmometer fitted with Amicon PM-10 membranes. Data from each solution were fit by stepwise regression with a three- or four-term polynomial in integral powers of total nondiffusible solute concentration in accordance with the general solution theory of McMillan and Mayer (1945, *J. Chem. Phys.* 13:276) as extended by Yamakawa (1971, *Modern Theory of Polymer Solutions*, Harper & Row, New York). The data display a high internal consistency, and the results correlate well with published molecular weights and exclusion data where available. Number average molecular weights calculated from the "first virial coefficients" are: BSA, 67,000 ± 11%; D110, 76,000 ± 11%, CHS, 39,000 ± 6%. Excluded volumes (in cubic centimeters per molecule) calculated from the "second virial coefficients" are: BSA, 0.97×10^{-18}; D110, 3.04×10^{-18}; CHS, 14.3×10^{-18}; BSA-D110, 6.8×10^{-18}; BSA-CHS, 7.8×10^{-18}. Uncertainty is about 30%. An empirical model for interpretation of calculated excluded volumes is proposed. It appears that CHS has the "largest" exclusion effect of the three molecules.

INTRODUCTION

There is at present no well-accepted theory by which one can calculate excluded volume from osmotic pressure data or interpret such results unambiguously. Moreover, there is still no way of obtaining sufficient data about particular intermolecular interactions to evaluate any theoretical expression for excluded volume derived from basic principles. A statistical mechanical theory of solutions formulated by McMillan and Mayer (1945) serves as a basis for calculating excluded volume from osmotic pressure data (Yamakawa, 1971). To go beyond the calculation of excluded volume to an explicit molecular interpretation then requires either details of intermolecular potentials, usually unobtainable, or the assumption of an empirical model. The latter approach has been followed in this paper. The application of the McMillan-Mayer (1945) theory also requires adequate Debye-Hueckel shielding of polyelectrolytes. The data in this study are demonstrated to be internally consistent without conflict with principle.

The usefulness of this modeling approach is established in part by the consistency of number average molecular weights of bovine serum albumin (BSA), dextran T110 (D110), and chondroitin sulfate (CHS), calculated from the reduced data and the model, with published information and data obtained by independent methods. The molecular weights obtained from mixed-solute solutions are consistent with those from single-solute solutions of the same nondiffusible solutes. The second virial coefficients and corresponding excluded volumes calculated for BSA in aqueous solutions of BSA and for D110 in aqueous solutions of D110 are in the range of values to be anticipated from published data (Scatchard et al., 1954; Landis and Pappenheimer, 1963; and Granath, 1958). Although this consistency does not prove the theory or establish the model, it is an important and necessary step.

Little data have been published concerning the osmotic properties, or directly related properties, of solutions containing CHS. Results obtained by Wasteson through gel column chromatography (1971) suggest that the CHS molecule is intermediate in configuration between rod and flexible chain at near-physiologic values of ionic strength and pH. The rod-like character would lead one to predict a nonideal osmotic behavior at relatively low concentrations of this molecule.

In this study osmotic pressure data are interpreted in terms of excluded volume. Two molecules cannot occupy the same space; the presence of molecule A excludes molecule B from a volume of space, and this is the excluded volume of molecule A with respect to molecule B. The exclusion effect is determined in either single- or mixed-solute aqueous solutions of BSA, CHS, and D110 with the objective of developing an approach for further application to solutions of biologic macromolecules, particularly the glycosaminoglycans and nonfibrous proteins of connective tissue. The osmotic pressure method is of particular interest among the methods for observing the second virial coefficient[1] and related excluded volume, because osmotic pressure is often employed by the physiologist to describe body fluids, the nature of the interstitium, and transcapillary exchange.

There have been such studies on solutions containing mixtures of nondiffusible components. Scatchard et al. (1954) examined the effects of pH and concentration on the second virial coefficient of the osmotic pressure expansion in solutions of human serum albumin and γ-globulins, but did not investigate excluded volume explicitly. Laurent and Ogston (1963) showed the dependence of osmotic pressure data taken from mixtures of hyaluronate and albumin on exclusion effects, but did not extensively investigate the relationship. For example, they did not calculate excluded volume on either a molecular or a molar basis or establish the parameter in different mixtures of the subject components. All of these investigators were handicapped by the use of heterogeneous and uncharacterized materials (i.e. the γ-globulins and the hyaluronate) and osmometers with long time constants, in the order of days.

[1] The second virial coefficient is defined as the coefficient of the second-order term of the solvent chemical potential expanded in powers of total nondiffusible solute concentration (Tanford, 1961).

METHODS AND MATERIALS

D110, BSA, and CHS were taken from single lots and human serum albumin (HSA) was obtained from a pooled blood bank sample. The D110 was described by number average and weight average molecular weights of approximately 76,000 and 110,000 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.; also note Granath, 1958). The sample of BSA was "fraction V" (Pentex Biochemical, Kankakee, Ill.). This sample produced a nearly identical chromatogram to that obtained from crystalline BSA (Armour, Chicago, Ill.) when both were eluted separately from a column of G-200. Both chromatograms indicated the presence of a minor polymerized component. The CHS (Sigma Chemical Co., St. Louis, Mo.) had a protein contamination of less than 0.03% as determined by amino acid analysis and exhibited number average and weight average molecular weights of 39,000 ± 10% and 50,000 ± 10% as determined by high-speed equilibrium ultracentrifugation. Chromatography on G-200 showed approximately the same elution volume as blue dextran, and on Sepharose 6B qualitatively the elution pattern predicted by extrapolations of earlier results obtained by Wasteson (1971) from a number of chondroitin sulfate preparations.

The principal solvent used was phosphate-buffered saline (0.15 M NaCl, 0.0019 M NaH_2PO_4, 0.0081 M Na_2HPO_4 in H_2O at pH 7.2). Sodium azide (0.02%) was a component of all solutions.

Solution concentrations were routinely monitored by refractometry. The refractometer was calibrated from standard solutions prepared according to measured solution volumes of weighed quantities of solute. The procedure appeared to result in more scatter for CHS than normal; therefore the calibration curve of refractive index vs. concentration for CHS was determined from averages of many measurements.

Osmotic pressure was measured with a temperature-controlled membrane osmometer (Instrumentation for Physiology and Medicine, Inc., San Diego, Calif.) with an average equilibration time of about 1 min, when fitted with Amicon PM-10 membranes (Amicon Corp., Lexington, Mass.). The reproducibility with this apparatus and membrane averaged about ±0.3 mm Hg. The reproducibility of the instrument was checked before each run with readings from solutions of HSA at concentrations of 5% and 10%, since these concentrations gave osmotic pressures approximately midrange on the two scales of the instrument. The means of 26 trials involving five different membranes at 23° C were 18.8 ± 0.5 mm Hg (SD) at 5% and 60.0 ± 1.3 mm Hg (SD) at 10%. When corrected to 37° C, these observations were within 6% of values predicted by the empirical curve obtained by Landis and Pappenheimer (1963) for HSA at 37° C and were identical (within the experimental reproducibility) to values obtained by W. H. Brown,[2] using the same model of osmometer, for both the identical samples of HSA and others prepared at these concentrations. Membranes were rejected which (*1*) gave significantly lower values of osmotic pressure for the HSA standards, or (*2*) had an equilibration time of greater than 3 min, or (*3*) could not maintain a steady pressure for at least 10 min after equilibration. Cross-checks were also performed with W. H. Brown on samples of solutions containing both D110 and BSA.

The osmotic pressure data from a series of solutions containing a given relative macromolecular solute composition were fit with a polynomial expansion in integral powers of concentration by means of a stepwise regression program (Program BMD02R, Dixon, 1970). Usually a polynomial of three terms was adequate to describe the data to within the experimental error.

The program generated the virial coefficients of this expansion together with the standard

[2] W. H. Brown, Department of Bioengineering, University of California, San Diego, is the designer of the osmometer used in this study.

errors of the coefficients. The uncertainties estimated for the number average molecular weights and excluded volumes calculated from these coefficients were compounded from these standard errors (Wilson, 1952, p. 272).

THEORY

According to McMillan and Mayer (1945), osmotic pressure data can be fit with a virial expansion in integral powers of concentration:

$$\pi = A_1C + A_2C^2 + 0(C^3) + \cdots, \tag{1}$$

where π is the osmotic pressure, and C is the total nondiffusible solute concentration.

They were able to show that the expansion representation is valid at low nondiffusible solute concentration provided no long-range forces act between the nondiffusible components. Both albumin and chondroitin sulfate are polyelectrolytes; however, observations were taken in the present study at the relatively high ionic strength of 0.15, and it will be assumed that there was sufficient Debye-Huekel shielding to satisfy the requirement.

The notation of Eq. 1 is used to describe the experimental data (see Results). Osmotic pressure is expressed in millimeters of Hg and concentrations of total nondiffusible solute in grams per 100 ml of solution.

It was necessary to extract number average molecular weights from the data for calculation of excluded volumes and evaluation of the validity of the general approach. The number average molecular weight in grams per mole can be evaluated from Eq. 2 expressed in the units of the data:

$$M_n = (0.75 \times 10^{-5})RT/A_1, \tag{2}$$

where R is the gas constant, and T is the absolute temperature. M_n, the number average molecular weight of total nondiffusible solute, is given by Eq. 3 for a solution containing two nondiffusible[3] solute components:

$$M_n = x_2M_2 + x_4M_4 = (w_2 + w_4)/(w_2/M_2 + w_4/M_4), \tag{3}$$

where M_J is the number average molecular weight of component J, and w_J is the weight fraction of nondiffusible solute consisting of component J, and X_J is the corresponding mole fraction.

Relative mole fractions were calculated from the number average molecular weights and relative weight fractions by Eq. 4:

$$X_J = (w_J/M_J)/(w_2/M_2 + w_4/M_4). \tag{4}$$

The number average molecular weight (see Eqs. 1 and 2) is the ideal, or low, concentration value.

The excluded volume and its relation to the second virial coefficient A_2 of the osmotic

[3] Even-number subscripts are traditionally reserved for the nondiffusible solutes (Casassa and Eisenberg, 1964).

pressure expansion (Eq. 1) can be found in the general solution theory of McMillan and Mayer (1945). This point was demonstrated by Yamakawa (1971). Using the notation and units of Yamakawa, one can rewrite the osmotic pressure expansion in the form

$$\pi/kT = \rho_s + \rho_s^2 B_2^\circ + O(\rho_s^3), \tag{5}$$

where k is Boltzmann's constant, ρ_s is the total nondiffusible solute concentration in molecules per milliliter of solution, and π is the osmotic pressure in dynes per square centimeter. The coefficient B_2° is a form of second virial coefficient alternative to that used in directly describing the experimental data (see Eq. 1) and is obtainable from the coefficient A_2 by conversion from one system of units to the other by Eq. 6:

$$B_2^\circ = (1.34 \times 10^7)(M_n^2/NRT)A_2, \tag{6}$$

where N is Avogadro's number.

For calculation of excluded volume from the coefficient B_2°, the expanded form shown in Eq. 7 is necessary:

$$B_2^\circ = -[x_2^2 b_{2,22}^\circ + x_2 x_4 b_{2,24}^\circ + x_4^2 b_{2,44}^\circ], \tag{7}$$

where $b_{2,JK}^\circ$ is the cluster integral of the set of two molecules, one of component J and one of component K.

For a solution of known composition in components 2 and 4, the coefficient B_2° is extracted from the data for the mixed-solutes. The values of $b_{2,22}^\circ$ and $b_{2,44}^\circ$ are determined from the appropriate sets of data for single-solutes. It is then possible to calculate the coefficient $b_{2,24}^\circ$, and from this coefficient, the excluded volume, u, by use of Eq. 8:

$$b_{2,JK}^\circ = -u/2. \tag{8}$$

The relationship between the coefficient $b_{2,JK}^\circ$ and excluded volume can be derived using a cluster integral, and this approach is outlined in the Discussion. In Eq. 8, the indices J and K always refer to nondiffusible components. In Eqs. 5–8 the superscript "°" indicates the constraint that the nondiffusible solute concentration be dilute.

For purposes of comparison with other types of molecular parameters, I have interpreted the results of the excluded volume calculations in terms of the equivalent sphere model defined by Eq. 9:

$$u = (4/3)\,\pi\,(D)^3, \tag{9}$$

where D is the average center-to-center separation of the two interacting "spherical" molecules.

RESULTS

Curve Fit and Data Scatter

For each mixture of macromolecular solute components, the best fit to the osmotic pressure data was obtained over two overlapping ranges in total nondiffusible solute

concentration: over the linear range at low concentration and over an expanded range including the linear portion. The two sets of computer-generated virial coefficients and associated standard errors obtained for each solute type are shown in Tables I and II. The data points and the computer-generated curves are shown in Figs. 1 *a* and *b*.

The best estimates of number average molecular weight come from the virial coefficient derived from the curve fit over the linear concentration range, judged by results from solutions containing BSA and D110 of known molecular weight. However, the difference between the two curve fits for each nondiffusible solute type was not significant over the lower concentration range when the standard error in the coefficients and the usual scatter in data were considered. Thus, the procedure followed in this paper was to calculate number average molecular weights from the lower concentration range linear coefficient A_1 and use the value of A_2 obtained from the fit over the expanded range in calculations of the coefficient B_2^* (see Eq. 6).

The data (Fig. 1 *a*) from solutions of BSA or D110, or BSA and D110 allowed direct calculation of the number average molecular weights of both BSA and D110, as described in the next part of the Results. There appeared to be an optimum concentration range of nondiffusible solution composition where smooth osmotic pressure data could be obtained from solutions containing CHS (see Fig. 1 *b*). Correspondingly, the number average molecular weight of CHS could most reliably be obtained from the data involving mixtures of CHS and BSA (of previously determined molecular weight). The "linear" region in the CHS data was not utilized in calculations because of the relatively wide scatter. The data for pure CHS were subsequently evaluated for consistency with the information from solutions of mixed BSA and CHS, as described in the next part of the Results.

Number Average Molecular Weights

Solutions of BSA, D110, and BSA and D110. From the low concentration range of each curve, the number average molecular weight of total nondiffusible solute was calculated for each of the five solute compositions listed in Table I, by means of Eq. 2, and substituted in Eq. 3. This procedure resulted in five equations in the unknown number average molecular weights of BSA and D110. The three equations involving the mixtures were then solved simultaneously in pairs, and three values for each of the two number average molecular weights were obtained. Each of these two sets of three values was averaged with the value obtained from the corresponding single nondiffusible solute solution. See Table III for results and comparison with independently established parameters.

Values for the relative mole fractions x_2 and x_4 were calculated by substitution of the measured values of the relative weight fractions w_2 and w_4 and the above values for number average molecular weights in Eq. 4. The results are given in Table I.

Solutions of CHS and Mixtures of CHS and BSA. The data from solutions containing CHS were not sufficiently precise to allow simultaneous calculation of number average molecular weight of both BSA and CHS by the procedure employed for

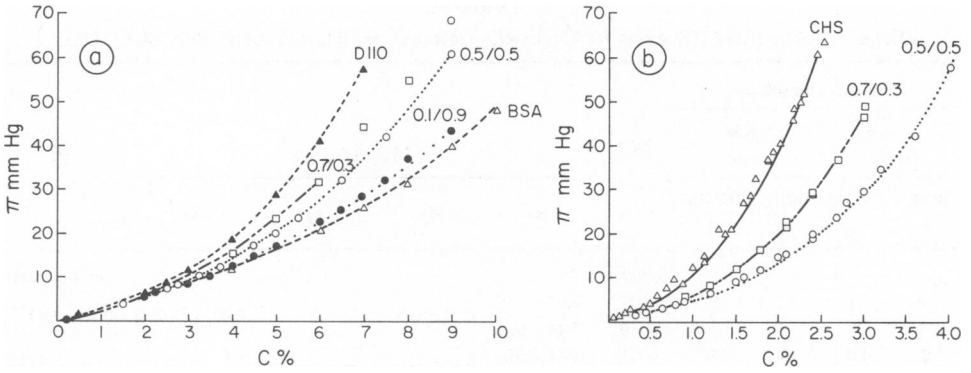

FIGURE 1*a* Osmotic pressure plotted against total nondiffusible solute concentration for solutions containing BSA and D110. The relative weight fractions corresponding to the five computer-generated curves are as follows: - - △ - -, BSA 1.0, D110 0; · · ● · ·, BSA 0.9, D110 0.1; · · · ○ · · ·, BSA 0.5, D110 0.5; - - - □ - - -, BSA 0.3, D110 0.7; - - - △ - - -, BSA 0, D110 1.0. D110, each point is the mean of 10 determinations with three membranes. BSA, each point is the mean of six determinations with two membranes. 30/70, each point is the mean of four determinations with two membranes. 50/50, each point is the mean of eight determinations with two membranes. 90/10, each point is the mean of six determinations with three membranes.

FIGURE 1*b* Osmotic pressure plotted against total nondiffusible solute concentration for solutions containing BSA and CHS. The relative weight fractions corresponding to the three computer-generated curves are as follows: —△—, BSA 0, CHS 1.0; · · · · ○ · · · ·, BSA 0.5, CHS 0.5; - - - □ - - -, BSA 0.3, CHS 0.7. CHS, each point is the mean of approximately 10 determinations with three membranes. Mixtures: each point is the mean of four to six determinations with two membranes. The curve for CHS is the "constrained" curve. The fit of the "constrained" curve is quite good, implying that the CHS data and the data from the mixed-solute solutions (CHS-BSA) are highly consistent with each other.

All of the above data (Fig. 1 *a* and *b*) could be fit with a power series of order 3 (see figure) over the expanded (i.e. entire) concentration range with three exceptions: where the nondiffusible solute consisted of BSA only and where it consisted of the mixtures of BSA and CHS. In these three exceptions, there were fourth-order contributions. The linear correlation coefficient between osmotic pressure and total nondiffusible solute concentration was at least 0.98 over the lower concentration region for all the data excepting those from solutions containing only CHS. Data from single-solute solutions of CHS had the greater scatter, as indicated by the large standard error in virial coefficients shown in Table II. At concentrations of CHS approaching 3%, the index of refraction used to monitor concentration was a less sensitive indicator of concentration than osmotic pressure itself. At the lowest levels of CHS concentration, however, the variable quantity of CHS that apparently tended to stick to the membrane, as observed visually, introduced observable uncertainty into the osmotic pressure results. There was less scatter in data from solutions containing both BSA and CHS.

the BSA-D110 system. Therefore, I substituted the value of number average molecular weight derived for BSA from the D110, BSA solution data in Eq. 3, written for each CHS-BSA mixture. As before, I used the virial coefficients from the linear, low concentration range to calculate M_n for each mixture from Eq. 2. The resulting values for M_4 (referring to CHS) with SD indicated were:

$$M_4 = 42{,}000 \pm 400 (w_2 = w_4 = 0.50);$$

$$M_4 = 38{,}000 \pm 200 (w_2 = 0.30; w_4 = 0.70).$$

TABLE I

VIRIAL COEFFICIENTS AND STANDARD ERRORS FOR D110 AND BSA SOLUTIONS

Nondiffusible solutes				ΔC*	Virial coefficients				B_2^*
Weight fraction		Mole fraction							
(BSA) w_2	(D110) w_4	(BSA) x_2	(D110) x_4		A_1	A_2	A_3	A_4	
				g/100 ml					*cm³/molecule*
1.0	0	1.0	0	0–2%	2.63 ± 0.03	0.12 ± 0.02	0	0.001 ± 0.0001	0.48×10^{-18}
				0–10%	2.44 ± 0.10				
0	1.0	0	1.0	0–1%	2.48 ± 0.06	0.29 ± 0.05	0.078 ± 0.005	0	1.52×10^{-18}
				0–7%	2.28 ± 0.12				
0.90	0.10	0.91	0.09	0–2%	2.62 ± 0.25	0.19 ± 0.06	0.012 ± 0.005	0	0.79×10^{-18}
				0–9%	2.18 ± 0.16				
0.50	0.50	0.53	0.47	0–2%	2.70 ± 0.09	0.24 ± 0.04	0.03 ± 0.003	0	1.10×10^{-18}
				0–9%	2.12 ± 0.11				
0.30	0.70	0.33	0.67	0–2%	2.47 ± 0.36	0.36 ± 0.16	0.032 ± 0.019	0	1.74×10^{-18}
				0–6%	2.01 ± 0.30				

*Concentration range used to construct a particular curve.

The mean osmotically determined number average molecular weight of CHS was, therefore, 40,000 ± 5%. This value is essentially identical to the ideal number average molecular weight determined by high-speed equilibrium ultracentrifugation. According to this ultracentrifugation determination,[4] the ideal number average and weight average molecular weights of this sample were 39,000 ± 10% and 50,000 ± 10%, respectively. The number average molecular weight averaged for the three determinations (two osmotic and one ultracentrifugal) was, therefore, 39,700 ± 6% (SD). In subsequent calculations involving number average molecular weight of CHS, this value was utilized.

Excluded Volume Calculations

I used the second virial coefficient (A_2) obtained by curve fitting over the expanded concentration range to calculate the quantity B_2^* from Eq. 6 for each nondiffusible solute composition, except that consisting of CHS only. These calculations also involved the number average molecular weights of total nondiffusible solute obtained by substitution of the relative mole fractions and number average molecular weights of the corresponding nondiffusible solute components in Eq. 3 (see the preceding part). The results are shown in Tables I and II. Solutions with high proportions of D110 and CHS had the large values of B_2^* and excluded volume. The average uncertainty in the excluded volumes estimated from the standard errors in the coefficients A_1 and A_2 for each mixture is about 30%.

Solutions of BSA, D110, and BSA and D110. To calculate excluded volumes,

[4]David C. Teller, Department of Biochemistry, University of Washington, Seattle, Wash. 98195.

TABLE II

VIRIAL COEFFICIENTS AND STANDARD ERRORS FOR BSA AND CHS SOLUTIONS

Nondiffusible solutes				ΔC*	Virial coefficients				B_2°
Weight fraction		Mole fraction							
(BSA) w_2	(CHS) w_4	(BSA) x_2	(CHS) x_4		A_1	A_2	A_3	A_4	
				g/100 ml					cm^3/molecule
0	1.0	0	1.0	0–2.5%	5.28 ± 1.3	6.0 ± 1.5	0.81 ± 0.43	0	7.17×10^{-18}†
				0–2.5%	4.64†	5.02†	1.40 ± 0.04†		
0.30	0.70	0.20	0.80	0–0.48%	4.24 ± 0.28				
				0–3.0%	3.39 ± 0.48	2.83 ± 0.35	0	0.16 ± 0.02	5.22×10^{-18}
0.50	0.50	0.37	0.63	0–0.48%	3.57 ± 0.17				
				0–4.0%	3.21 ± 0.33	1.72 ± 0.18	0	0.07 ± 0.01	3.85×10^{-18}

*Concentration range used to construct a particular curve.
†Inferred from BSA/CHS mixture data.

I performed an intermediate calculation to obtain the coefficients $b_{2,JK}^\circ$ (see Eqs. 7 and 8). The coefficients $b_{2,22}^\circ$ and $b_{2,44}^\circ$ were obtained directly from the respective coefficients B_2° derived from single-nondiffusible-solute solutions of BSA and D110 according to Eq. 7. From the values of B_2° for the mixtures and the coefficients $b_{2,22}^\circ$ and $b_{2,44}^\circ$, the quantities $b_{2,24}^\circ$ were obtained for the D110-BSA mixtures. The resulting values of $b_{2,24}^\circ$ were averaged, and a value of excluded volume, u, for the D110-BSA pair was calculated from this average. Excluded volumes in the single-solute solutions (from $b_{2,22}^\circ$ and $b_{2,44}^\circ$) and in the D110-BSA solutions are given in Tables III and IV. Note that the excluded volume per molecule (D110 with respect to BSA or vice versa) is largest in the mixed system (BSA-D110).

Solutions of CHS and Mixtures of CHS and BSA. Eq. 7 was evaluated for the two mixtures ($w_2 = w_4 = 0.50$) and ($w_2 = 0.30; w_4 = 0.70$). In this section the subscript "2" designates BSA, as usual, and the subscript "4" designates CHS. The calculated values for the mole fractions x_2 and x_4 for these mixtures were substituted in the equation. The actual value of $b_{2,22}^\circ$ for BSA was that extracted from the BSA-D110 system. The resulting pair of simultaneous equations in two unknowns ($b_{2,24}^\circ$ and $b_{2,44}^\circ$) was then solved, yielding:

$$b_{2,24}^\circ = -3.9 \times 10^{-18}\,\text{cm}^3/\text{molecule}; \quad b_{2,44}^\circ = -7.2 \times 10^{-18}\,\text{cm}^3/\text{molecule}.$$

The value of B_2° for single-nondiffusible-solute solutions of CHS, as inferred from the mixture results, could then be calculated from Eq. 7 and was found to be $7.2 \times 10^{-18}\,\text{cm}^3/\text{molecule}$.

The values of the coefficients A_1 and A_2 were then calculated from Eqs. 2 and 6, based solely on the mixture data, for single-nondiffusible-solute solutions of CHS.

Assuming the above inferred values for A_1 and A_2, I calculated the value of A_3

TABLE III

OSMOTIC SEPARATION PARAMETER *D:* COMPARISON WITH STOKES RADIUS AND RADIUS OF GYRATION (SINGLE NONDIFFUSIBLE SOLUTE DATA)

Solute	π Data*		Standard Radii*		π Data†	Standard§	
	u	$D/2$	Stokes	Gyration	M_n	M_n	M_w
BSA	0.97×10^{-18}	31	36‖	30¶	66,500	67,000	—
D110	3.04×10^{-18}	45	45–100**	100‡‡	76,000	76,000	110,000
CHS	14.3×10^{-18}	75	65§§	—	40,000	40,000	50,000

*Radii in angstroms; excluded volume, u, in cubic centimeters; uncertainty in the excluded volume is about 30%.

‡Uncertainty is 12% or less.

§The values for BSA and CHS are from ultracentrifugation; the uncertainty is about 10%.

‖Diffusion (Wagner and Scheraga, 1956).

¶X-ray scattering (Anderegg et al., 1955).

**Radius calculated from second virial coefficient (light scattering), gives lower limit; upper limit obtained from viscosity measurements (Granath, 1958).

††Light scattering (Granath, 1958).

§§Gel chromatography (Wasteson, 1971).

giving the best fit to the CHS data with the regression program (see Table II, numbers with dagger [†]).

The corresponding constrained curve for CHS is shown together with the experimental data points in Fig. 1 *b*.

The values of the three virial coefficients obtained by direct fit of the data, shown in Table II, can be compared with the coefficients obtained indirectly from the mixtures (indicated with a dagger). It can be seen that the two sets of coefficients are consistent although not identical.

Osmotic Parameter, D/2

The parameter D is illustrated for the example of hard spheres in Fig. 2. In that situation D is given by the sum of the molecular radii, R_1 and R_2. In the general case,

TABLE IV

OSMOTIC SEPARATION PARAMETER *D:* COMPARISON WITH AVERAGES OF STOKES RADIUS* AND RADIUS OF GYRATION* (MIXED NONDIFFUSIBLE SOLUTE DATA)

Solute	π Data†		From Stokes radius§		From radius of gyration§	
	u	$D/2$	$\frac{1}{2}(R_{s_2} + R_{s_4})$	$(R_{s_2} \cdot R_{s_4})^{1/2}$	$\frac{1}{2}(R_{g_2} + R_{g_4})$	$(R_{g_2} \cdot R_{g_4})^{1/2}$
BSA-D110	6.8×10^{-18}	59	40–68	40–60	65	55
BSA-CHS	7.8×10^{-18}	60	50	48	—	—

*See Table III for values.

†Dimensions of excluded volume, u, are cubic centimeters; uncertainty in u is about 30%.

§R_{s_2} and R_{g_2} are Stokes radius and radius of gyration, respectively, for BSA; R_{s_4} and R_{g_4} are those for D110 CHS. Range for BSA-D110 corresponds to range in Stokes radius for D110 (Table III). Linear dimensions are in angstroms.

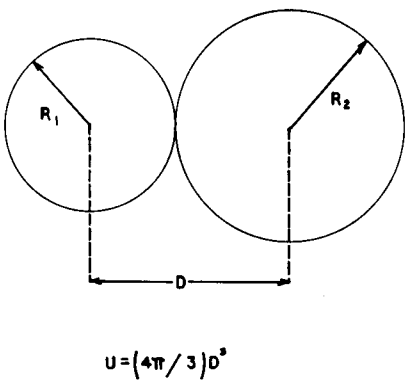

Figure 2

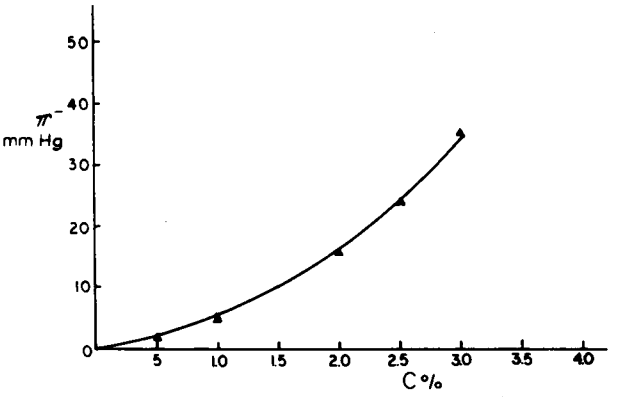

Figure 3

FIGURE 2 The parameter D is illustrated for the example of a solution of two nondiffusible solute components which can be represented by hard spheres of radii R_1 and R_2. In this instance $D = R_1 + R_2$.

FIGURE 3 Osmotic pressure plotted against total nondiffusible solute concentration. The nondiffusible solute components are D110 and CHS present in the relative weight fractions $w_2 = w_4 = 0.50$. Each point is the average of four determinations with a single membrane. The curve is the "constrained" curve.

I am defining D as twice an average molecular radius and will compare $D/2$ with a geometric mean radius $(R_1 R_2)^{1/2}$—and an arithmetic mean radius $(R_1 + R_2)/2$.

The osmotic parameter, $D/2$, was calculated from excluded volumes by Eq. 9 for the macromolecules BSA, D110, and CHS in solution as the single nondiffusible solute and for the mixed pairs BSA-D110 and BSA-CHS. The results are shown in Tables III and IV. The value of $D/2$ is largest for CHS for the single-solute solutions. BSA and D110 are seen to be comparable in "size" in their single-solute solutions. BSA has about the same interaction parameter with respect to either CHS or D110, as shown by the results from the mixed-solute solutions.

Values of Stokes radius and radius of gyration for BSA, D110, and CHS were included in Table III where available. The arithmetic and geometric means of these radii are given in Table IV for the two classics (BSA-D110 and BSA-D110) of mixture. (The rationale for introducing these hydrodynamic radii is presented in the Discussion.) Since the overall standard errors in the determinations of $D/2$ are about 30%, $D/2$ in the single-solute solution is essentially undistinguishable from the corresponding Stokes radius for either BSA, D110, or CHS but smaller than the radius of gyration for D110 (Table III). The values of $D/2$ for the two classes of mixture are within the ranges defined by the standard averages of the hydrodynamic radii; however, one cannot make a more refined comparison because of experimental error.

Consistency Check Involving D110-CHS

Osmotic pressure measurements of mixed-solute solutions of D110 and CHS with relative weight fractions 0.50 and 0.50, respectively, were conducted for evaluation of the

method developed from data from the other combinations of nondiffusible solutes reported above (see Fig. 3).

I calculated the first virial coefficient from Eq. 2, assuming a value for the number average molecular weight of the total nondiffusible solute calculated by substituting the above values of w_2 and w_4 and values for M_2 and M_4 previously obtained for D110 and CHS in Eq. 3. The resulting value for A_1 was 3.55 ± 0.10.

To estimate from theory a value for the second virial coefficient, A_2, I first hypothesized a value for the osmotic parameter, $D/2$. The value of $D/2$ assumed as geometric mean of Stokes radii for D110 and CHS (Table IV) or as geometric mean of the individual values of $D/2$ for CHS or D110 was 56 ± 5 Å. The value of $b^{\circ}_{2,24}$ was then calculated from Eqs. 8 and 9. The value of A_2 resulting from this assumption for $D/2$ is 1.11. This value for A_2 was obtained from Eqs. 6 and 7 in which the values previously obtained for $b^{\circ}_{2,22}$ representing D110 and $b^{\circ}_{2,44}$ representing CHS were substituted. The substituted values of x_2 and x_4 were those calculated by use of previously obtained values of M_2 and M_4, of 76,000 and 39,600, respectively.

From the data and the above values of A_1 and A_2, the best least-squares value for A_3 was calculated. The resulting forced, or constrained, curve is shown in Fig. 3. As shown in Fig. 3, the data for the D110-CHS solutions could be fit relatively well, thus validating the general approach. The deviations from the curve can be easily explained on the basis of the uncertainties in the values for A_1 and A_2.

DISCUSSION

Excluded Volume and D/2

The intermolecular excluded volume has been shown to be equal to the following cluster integral for a solution of a single nondiffusible solute (Yamakawa, 1971; Hill, 1960):

$$u = \int \{1 - \exp[-V_{12}(S_{12})/kT]\} d\bar{S}_{12},$$

where $\bar{S}_{12}$ is the vector between the centers of mass of two interacting molecules, and $V_{12}(S_{12})$ is the average intermolecular potential (assumed to be of relatively short-range nature).

The coefficient $b^{\circ}_{2,JK}$ (see Eq. 8) is also proportional to a cluster integral of the same form (Yamakawa, 1971):

$$b^{\circ}_{2,JK} = (-1/2) \int \{1 - \exp[-V_{12}(S_{12})/kT]\} d\bar{S}_{12},$$

where the interacting molecules being to two different components. The coefficient $b^{\circ}_{2,JJ}$ has been explicitly identified with excluded volume for the nondiffusible component in single-solute solution (Yamakawa, 1971). I believe that my paper is the first account explicitly extending the identification of $b^{\circ}_{2,JK}$ to excluded volume in the binary solution (Eq. 8) and applying it to interpret osmotic pressure data. The cluster-integral approach emphasizes the concept that excluded volume is a function of all forces acting between a pair of molecules, including solvent modifications. The de-

tailed nature of the potential need not necessarily be specified; e.g., excluded volume is not defined on purely steric grounds. This formulation is advantageous in situations where the intermolecular potential is ill-defined. In the simplified example of a solution of uncharged, hard spheres of radius R, the cluster-integral expression for excluded volume reduces to the anticipated form: $u = (32/3)\pi R^3$ (Hill, 1960).

The definition of thermodynamic excluded volume requires that the interacting molecular components either have identical shapes or be assigned analogous equivalent shapes. The simplest approach and a useful point of departure seemed to be the assumption of equivalent spherical configurations, and hence the parameter $D/2$. Note that D does not describe a purely steric interaction since the excluded volume from which D is defined includes the effect of all acting intermolecular forces.

The comparison of the parameter D (Eq. 9) with the Stokes radius and radius of gyration is not meant to imply conceptual equivalence. Excluded volume is a more complex concept than the hydrodynamic equivalent sphere from which the Stokes radius is calculated. It is a function of intermolecular interactions, whereas the hydrodynamic measurements defining the Stokes radius are made in, or extrapolated to, dilute solutions where intermolecular interactions are relatively insignificant (Tanford, 1961). The Stokes radius is a function of molecular shape and, therefore, provides a comparison of interest. For random coil polymers, the difference between $D/2$ and the Stokes radius and the radius of gyration theoretically would reflect the "compressibility" of the molecule (Tanford, 1961). There was empirical correspondence between $D/2$ and the ranges spanned by arithmetic and geometric means of these quantities for BSA-D110 and BSA-CHS (Table IV).

Albumin

Osmotic pressure data points taken from solutions of HSA (pooled blood bank sample) closely approximate values predicted for HSA by the empirical Landis-Pappenheimer equation adjusted to 23°C: at 5% and 10%, the respective values of 18.8 ± 0.5 mm Hg and 60.0 ± 1.3 mm Hg were obtained where values of 19.1 mm Hg and 58 mm Hg are predicted by the Landis-Pappenheimer equation. This close agreement presents an interesting contrast with data from the solutions of BSA, fraction V. The BSA data points (consistent with cross-checks by W. H. Brown) lie on a lower curve, as reflected by a somewhat lower value of A_2 (0.12 as compared with 0.18, Landis and Pappenheimer, 1963). One might interpret this observation in terms of conformational or other changes occurring during purification and freeze-drying of the BSA, fraction V. Osmotic pressures developed by solutions of Armour crystalline BSA agreed closely with the data from solutions of BSA, fraction V.

D110

The value of second virial coefficient (0.29 ± 0.05) extracted from the data (Table I, column 6) is comparable with the value obtained from light-scattering experiments (Granath, 1958), since the value of the second virial coefficient determined by light scattering is theoretically twice that obtained from osmotic pressure measurements.

The value quoted by Granath for Pharmacia dextran of weight average mol w 110,000, when recalculated in units (millimeters Hg per gram percent) is about 0.72 ± 10%. The value of $D/2$ calculated from my data is smaller than the value of Stokes radius[5] calculated from viscosity data and less than the radius of gyration, as expected on the basis of molecular compressibility (Table III).

CHS

According to the extrapolated results from Wasteson (1971), CHS with weight average mol wt 50,000 would have approximately the same elution behavior on G-200 and Sepharose 6B as Ficoll with a Stokes radius of 65 Å. My data together with the model define an interaction radius ($D/2$) of about 75 Å in single-solute solutions of CHS. The value of the parameter $D/2$ calculated from the excluded volume of CHS is significantly larger than that of either D110 or BSA, which have somewhat larger molecular weights (Table III). This observation may result from steric factors, reflecting a rod-like configuration for the CHS molecule (Wasteson, 1971). It is apparent both from the highly nonlinear character of the data and the magnitude of the calculated excluded volume, u, that CHS is almost twice as effective as D110 in excluding BSA: CHS ($M_n \sim 39{,}000$) excludes BSA to the same degree as D110 ($M_n \sim 76{,}000$).

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[5]Calculated by use of the model of Laurent and Killander (1964).

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