review

Application of osmometric methods to the study of lipids, lipoproteins, and apolipoproteins

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Abstract Membrane and vapor pressure osmometry are two colligative methods that can be useful in lipid research. The former method can be used to study proteins or other macromolecules whose molecular weight lies between 20,000 to 1,000,000. Vapor pressure osmometry is useful with smaller molecules having a molecular weight of 10,000 or less. These techniques can be used in aqueous or nonaqueous solutions. They are rapid, precise, nondestructive, and require relatively small amounts of material. These techniques provide information about the state of aggregation and also about interactions of lipids, lipoproteins, and apolipoproteins in solution. We will show how membrane osmometry can be used to study solutions of lipoproteins and apolipoproteins. The application of vapor pressure osmometry to the study of biologically important lipids such as cholesterol, cholesteryl esters, and bile salts is shown.—**Adams, E.T., Jr.** Application of osmometric methods to the study of lipids, lipoproteins, and apolipoproteins. *J. Lipid Res.* 1986. **27:** 1233–1247.

Supplementary key words membrane osmometry • vapor pressure osmometry • protein molecular weights • state of aggregation • interactions of lipids, lipoproteins, and apolipoproteins in solution • cholesterol

INTRODUCTION

A colligative property is one that depends primarily on the number of molecules concerned and not on their nature.[1] Colligative properties of solutions are also known as osmotic properties. Two of these properties, membrane osmometry (1–3), MO, and vapor pressure osmometry, (3), VPO, are useful in the study of lipids, lipoproteins, and apolipoproteins. For solutions of smaller molecules (molecules whose molecular weight is less than 10,000) such as cholesterol, cholesteryl esters, bile acid methyl ester, bile salts, etc., VPO is a very useful technique for obtaining molecular weights, studying self-associations or mixed associations, and detecting the effect of various solvents on the state of aggregation of the small solutes (3). Similar studies can be done on larger solutes (molecules having a molecular weight between 10,000 to 1,000,000), such as apolipoproteins or lipoproteins, using MO (1–3). The theory for both techniques is quite similar, and the procedures are not that difficult. Most biochemists know how to use a syringe, so it should be rather easy for them to become proficient with a VPO apparatus. This technique requires only small amounts of material. The development of high speed membrane osmometers, in which osmotic equilibrium is attained in a short time of 5–20 min instead of hours or days, has revitalized this method. Both techniques can be used with aqueous or nonaqueous solutions, and both techniques can be done at various temperatures, including 37°C (body temperature). In order to perform VPO one must have a nonvolatile solute, and the solution must usually contain only one solute. We will show ways that one can do VPO studies in aqueous solutions containing more than one solute (4).

Membrane osmometry can be performed in aqueous solutions containing buffers and supporting electrolytes along with the protein of interest, or MO can be done in solutions containing one or a mixture of solvents (1–3). With the development of a reduction insert, small volumes (50 μl) are needed for each individual rinse or measurement.

Why use osmometric measurements? They are rapid, require small amounts of material, and the methods are nondestructive. With a nonassociating solute one can determine the molecular weight (M) or the number average molecular weight (M_n) in a day. The theory is much sim-

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Abbreviations: AK, attenuated equilibrium constant; apoA-I, apolipoprotein A-I; apoB, apolipoprotein B; ATP, adenosine triphosphate; CD, circular dichroism; CH, cholesterol; CHS, cholesteryl stearate; CHP, cholesteryl palmitate; EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoprotein; MeC, methyl cholate; MeLC, methyl lithocholate; MO, membrane osmometry; ORD, optical rotary dispersion; SEK, sequential, equal equilibrium constant; SM, semipermeable membrane; VPO, vapor pressure osmometry.

[1] The literature search for this review was completed on May 31, 1986.

pler than that for ultracentrifugation (5) or light scattering (6, 7). Although one has to know the concentration of the solute of interest for all three methods, it can be determined by the most convenient method. One does not necessarily have to determine the refractive index increment, which is required for light scattering (6, 7) and sedimentation equilibrium experiments when refractometric (5) (Rayleigh or schlieren) optics are used. It is not necessary to know the partial specific volumes or the density increments in order to obtain M or M_n. Dust is no problem; it is a serious problem in light scattering, since very small amounts of dust can cause very large errors (7, 8). Pressure effects, which must be corrected for in sedimentation equilibrium experiments in nonaqueous solutions, are not a problem in VPO or MO. VPO and MO determinations can be made at 37°C, human body temperature, as well as other temperatures. Unless the ultracentrifuge has been modified for high temperature operation, it is difficult to do ultracentrifugation at temperatures above 30°C because drive oil fogs the upper and lower schlieren lenses; most ultracentrifuges are not so modified.

We will show results obtained with osmometric studies on large and small molecules under various conditions: homogeneous, nonassociating solutes; heterogeneous, nonassociating solutes; mixed associations between two different solutes; self-associating solutes. Some of the examples will deal with published material and some other examples will cover original investigations. It is hoped that this review will stimulate interest in these techniques for those who engage in lipid research.

BACKGROUND INFORMATION

Osmotic pressure (1–3)

The principle of a membrane osmometer is illustrated in **Fig. 1.** At constant temperature T, a solution containing a macromolecular solute (side 2) is separated from the solvent or buffer solution (side 1) by a semipermeable membrane (labeled 3). If the pressure on both sides were the same (P), then solvent will flow from side 1 to side 2, since the chemical potential of the solvent is higher in side 1 than it is in side 2. This solvent flow would cause a rise in the liquid level in side 2 until the increase in the hydrostatic pressure in side 2 became sufficiently large to stop the flow of solvent from side 1. The semipermeable membrane will allow solvent and other small diffusible components (supporting electrolyte components such as KCl or NaCl and buffer components) to pass through the membrane, but it will restrain the macromolecular solute. One can prevent the flow of solvent from side 1 to side 2 by increasing the pressure on side 2 from P to P + Π. This excess pressure Π is the osmotic pressure. It is also

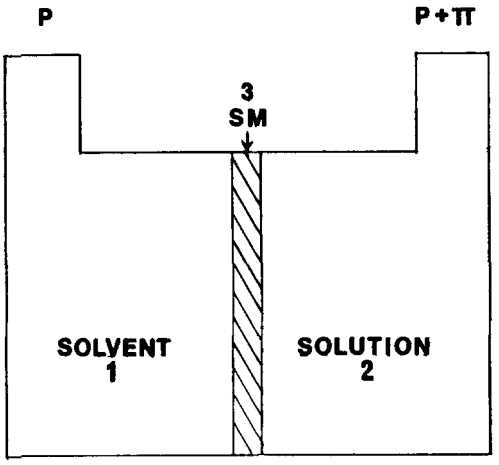

Fig. 1. Principle of a membrane osmometer. At constant temperature (T) a solution (2) is separated from the solvent or buffer (1) by a semipermeable membrane (3). When the pressure on both sides is the same, solvent will flow from side 1 to side 2 until the increased hydrostatic pressure on side 2 stops the solvent flow. When the difference in pressure between the two sides is Π, no solvent will flow from side 1 to side 2. This excess pressure Π is known as the osmotic pressure.

possible to prevent the flow of solvent from side 1 to side 2 by decreasing the pressure on side 1 from P to P − Π. Thus, as long as the pressure difference between the two sides is Π, no solvent will flow from side 1 to side 2. When the pressure on side 2 exceeds P + Π, then solvent will flow from side 2 to side 1; this is sometimes called reverse osmosis. A sketch of the basic theory of osmotic pressure is given in the Appendix. For more details on the theory one should consult refs. 1–3.

The osmotic pressure equation can be written as (1–3)

$$\Pi\bar{V}_0/RT = -\ln a_0 \qquad Eq.\ 1)$$

or as

$$\Pi\bar{V}_0/RT = -g \ln x_0. \qquad Eq.\ 2)$$

Here Π is the osmotic pressure, R is the gas constant, T is the absolute temperature, $\bar{V}_0$ is the partial molar volume of the solvent whose mole fraction is x_0 and whose activity is a_0, and g is the osmotic coefficient of the solvent. It is customary to refer to g simply as the osmotic coefficient, and g is defined by

$$g = \Pi(\text{real})/\Pi(\text{ideal}). \qquad Eq.\ 3)$$

For an ideal solution, g = 1 and $a_0 = x_0$. Note that from eq. *1* it is possible to obtain the activity of the solvent, and when x_0 is known, then g is known from eq. *2*. Equation *2* will be used in developing equations for the analysis of osmotic pressure data.

Vapor pressure osmometry (3, 9–11)

When vapor and solvent are in equilibrium at constant T,

$$\mu_0(\text{gas}) = \mu_0(\text{liq}). \qquad Eq.\ 4)$$

Fig. 2 shows two thermistor probes, A and B, which are connected to a very sensitive Wheatstone bridge circuit (12). When a drop of solution is placed on thermistor bead A and a drop of solvent is placed on thermistor bead B, solvent vapor will condense on bead A since the chemical potential of the solvent in the solutions is less than that of the pure solvent. The change of phase from vapor to liquid will release heat and cause a slight temperature difference between the two thermistor beads, and this temperature difference will be detected as an imbalance in the Wheatstone bridge.

In vapor pressure osmometry, one detects the temperature difference (ΔT) between the thermistors (see Fig. 2) by the resistance change (Δr) or by the microvolts imbalance (E) on the Wheatstone bridge circuit of the vapor pressure osmometer. The quantity ΔT depends on the number of solute particles in solution; under ideal solution conditions this is given by c/M when the solute is homogeneous and nonassociating, or by c/M_n when the solute is heterogeneous and nonassociating. Under nonideal conditions these become c/M_{app} and c/M_{na}, respectively. Since E (or Δr) is proportional to ΔT, one notes that

$$E = K_{vp}(c/M_{na}) \qquad Eq.\ 5)$$

when the sample is heterogeneous and nonassociating; here M_{na} is the apparent number average molecular weight. When the solute is homogeneous and nonassociating, one obtains

$$E = K_{vp}(c/M_{app}). \qquad Eq.\ 6)$$

Here M_{app} is the apparent molecular weight, c is the total solute concentration, and K_{vp} is an operational instrument constant whose value depends on the temperature and the solute–solvent combination. K_{vp} is determined from VPO experiments, at the same temperature and with the same solvent, on a nonvolatile solute of known molecular weight. With nonaqueous solvents, benzil is often used as a standard. With aqueous solutions, sucrose, mannitol, or some other nonvolatile solute can be used as a standard. From measurements of E versus c or E/c versus c one notes

$$\lim_{c\to 0}\frac{dE}{dc} = K_{vp}/M_{STDD} \qquad Eq.\ 7)$$

or

$$\lim_{c\to 0} E/c = K_{vp}/M_{STDD} \qquad Eq.\ 8)$$

Here M_{STDD} is the molecular weight of the standard; since this is known, one can obtain K_{vp}. **Fig. 3** shows a plot of E/c versus c for aqueous mannitol solutions at 37°C; the plot is virtually horizontal which indicates that the solution is ideal (see eq. *15*). The intercept of this plot gives K_{vp}/M_{STDD}; here M_{STDD} = 182.17 g/mole for mannitol so that K_{VP} = 43.72.

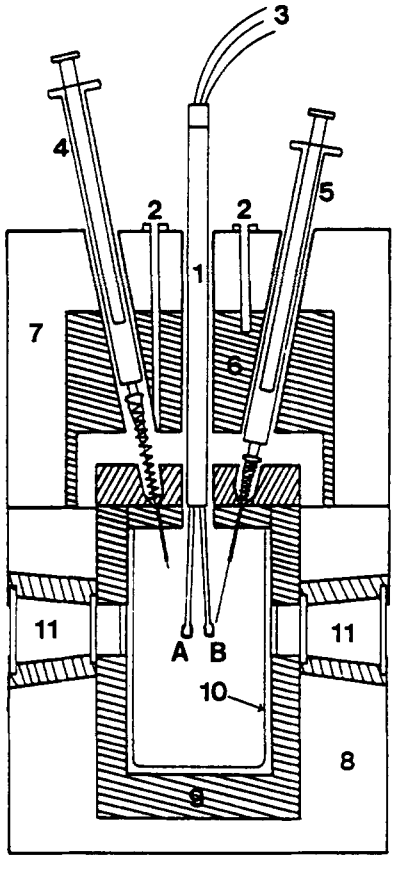

Fig. 2. Schematic of the cell of Knauer vapor pressure osmometer. The cell is maintained at constant temperature. The numbers refer to the following items: 1, thermistor probe with two thermistors (A and B); 2, opening for measuring cell temperature with a thermometer; 3, thermistor connections to the Wheatstone bridge; 4, syringe in rest position; 5, syringe in position for applying a drop on the thermistor; 6, thermostatted metal block for warming syringes; 7, glass wool-insulated upper half; 8, glass wool-insulated lower half; 9, thermostatted aluminum block for measuring cell; 10, glass liner for measuring cell and solvent reservoir; 11, viewing windows. Taken from ref. 12, Fig. 1.

HOMOGENEOUS, NONASSOCIATING SOLUTES (1–3)

When there is only one nonassociating solute present, $x_0 = 1 - x$, where x is the mole fraction of solute. Thus eq. *2* becomes (1–3)

$$\frac{\Pi\bar{V}_0}{RT} = -g \ln x_0 = -g \ln (1 - x_1)$$

$$= -g\left(-x_1 - \frac{x_1^2}{2} - \cdots\right) \cong g x_1. \qquad Eq.\ 9)$$

In dilute solutions

$$x_1 \cong \frac{n_1}{n_0} = \frac{c_1\bar{V}_0}{M_1} \qquad Eq.\ 10)$$

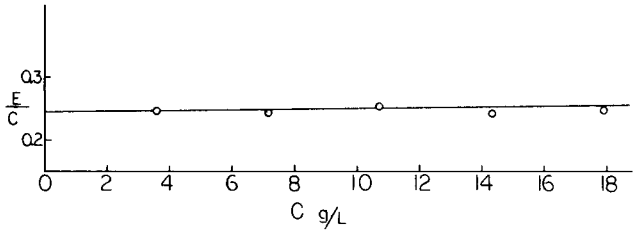

Fig. 3. Vapor pressure calibration plot. Shown are plots of E/c versus c for aqueous mannitol solutions at 37°C. The molecular weight of mannitol is 182.17; one can obtain K_{VP} from the intercept, since the intercept is $K_{VP}/182.17$ in this case. The horizontal nature of the plot indicates that the solution is virtually ideal.

where c_1 is the concentration of the solute in g/l, M_1 is the solute molecular weight, and $n_0 = 1/\bar{V}_0$ for $\bar{V}_0$ in 1/mol. Insertion of eq. *10* into eq. *9* leads to

$$\frac{\Pi}{RT} = g(c_1/M_1). \qquad Eq.\ 11)$$

For ideal solutions g = 1, so that

$$\frac{\Pi}{RT} = c_1/M_1. \qquad Eq.\ 12)$$

For nonideal solutions, g, the osmotic coefficient, can be expressed as a power series in c_1; thus

$$g = 1 + \frac{BM_1c_1}{2} + \cdots \qquad Eq.\ 13)$$

and eq. *11* becomes

$$\frac{\Pi}{RT} = \frac{c}{M_{app}} = \frac{c}{M} + \frac{Bc^2}{2} \qquad Eq.\ 14)$$

when the solution is not too nonideal. Here $M = M_1$, the solute's molecular weight. Under ideal solution conditions, g = 1 and B = 0. Here M_{app} is defined by

$$\frac{1}{M_{app}} = \frac{1}{M} + \frac{Bc}{2}. \qquad Eq.\ 15)$$

Thus a plot of $1/M_{app}$ versus c will have an intercept of 1/M and a slope of B/2, where B is the osmotic pressure second virial coefficient and is related to g by eq. *13*. When the solution is more nonideal, a plot of 1/M versus c will show upward curvature instead of a straight line.

Fig. 4 shows plots of Π/c versus c for a lipoprotein and two apolipoproteins in 10 mM Tris buffer (1 mM trishydroxyaminomethane plus sufficient HCl to reach pH 8.0, 5 mM NaN_3, and 1 mM disodium ethylenediaminetetraacetate [Na_2EDTA]) at pH 8 and 37°C. Plot A is a graph of Π/c versus c for rhesus monkey apolipoprotein A-I; here the buffer also contained 2 M guanidinium hydrochloride. Note that the plot indicates that there is no self-association, and the slight, positive slope suggests that the solution is very slightly nonideal. It would be ideal if the plot were horizontal. The value for M was 27.6×10^3 g/mol, which is about the same as M (28,014) for human apoA-I (13). The reduction insert was used in these experiments. All dilutions and measurements were done with a stock solution of 2 ml whose concentration was 10.8 mg/ml.

How well does the osmometer perform with higher molecular weight solutes? Fig. 4B shows a plot of Π/c versus c for canine high density lipoprotein. The inclined straight line indicates that the solution is nonideal and nonassociating; here a value of $M = 2.13 \times 10^5$ g/mol was obtained. Using sedimentation equilibrium experiments, Edelstein, Halari, and Scanu (14) obtained $M = 2.06 \times 10^5$ g/mol. The agreement between the two results is gratifying. Again the reduction insert was used. Measurements have also been performed on human apolipoprotein B in 6 M guanidinium hydrochloride solutions, as shown in Fig. 4C. Clearly, the solution is nonideal. The value of M was 4.00×10^5 g/mol; this compares favorably with values of 3.70×10^5 g/mol obtained by sedimentation equilibrium and 3.87×10^5 g/mol obtained by a combination of diffusion and sedimentation velocity experiments (15). **Table 1** lists the value of M and B_{OS} obtained in these experiments. Experiments like those shown in Fig. 4 can be done in 1 working day. The plots in Fig. 4 were obtained by a linear regression analysis using procedures described by Fischer (16); the values of M_n, B_{OS}, and the errors were obtained from the regression analysis (see Table 1). For more details on analyzing the osmotic pressure data and on how to convert osmotic pressure in cm of solvent to atmospheres, one should consult pp. 92–103 of ref. 1 or pp. 94–96 of ref. 3.

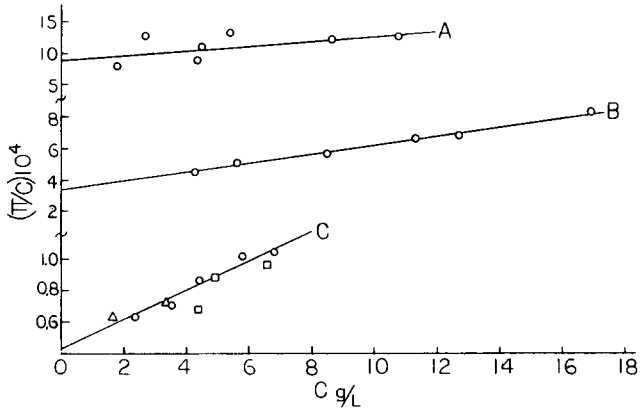

Fig. 4. Membrane osmometry. Plots of Π/c versus c at 37°C for the following solutions: A, rhesus monkey apoA-I in 10 mM Tris buffer, pH 8 (this buffer solution also contains 2 M guanidinium hydrochloride); B, canine HDL in 10 mM Tris buffer; C, human apoB in 6 M guanidinium hydrochloride (○ first preparation, □ second preparation, and △ third preparation with 0.01 M dithiothreitol added). Note that all three plots indicate that these solutions are nonideal and nonassociating.

TABLE 1. Molecular weights of three proteins obtained from the Knauer high-speed membrane osmometer using a reduction insert[a]

Protein[b]	M (g/mol)[c]	B_{OS} ($l \cdot mol \cdot g^{-2}$)	Solvent
Rhesus monkey apoA-I	$(2.76 \pm 0.34) \times 10^4$	$(2.5 \pm 1.3) \times 10^{-6}$	10 mM Tris (Tris + HCl), 5 mM NaN_3, 1 mM EDTA, 2 M guanidinium hydrochloride; pH 8.0
Canine high density lipoproteins	$(2.06 \pm 0.06) \times 10^5$	$(2.13 \pm 0.01) \times 10^{-6}$	10 mM Tris (Tris + HCl), 5 mM NaN_3, 1 mM EDTA; pH 8.0
Human apoB	$(4.00 \pm 0.40) \times 10^5$	$(7.1 \pm 1.1) \times 10^{-7}$	6 M guanidinium hydrochloride

[a] Temperature = 310.2 K (37°C).
[b] Amicon XM-10 membrane used.
[c] Obtained by linear regression analysis.

Fig. 5A shows plots of E/c versus c for VPO experiments on two cholesteryl esters in $CHCl_3$ solutions at 37°C. Similar plots for these esters in CCl_4 solutions at 37°C are shown in Fig. 5B. Note that in both cases these esters behave as nonassociating solutes, whereas cholesterol associates in both solvents. In $CHCl_3$ solutions at 37°C, the two esters appear to behave ideally, whereas in CCl_4 solutions at 37°C the CHP exhibits nonideality and the CHS seems to behave ideally.

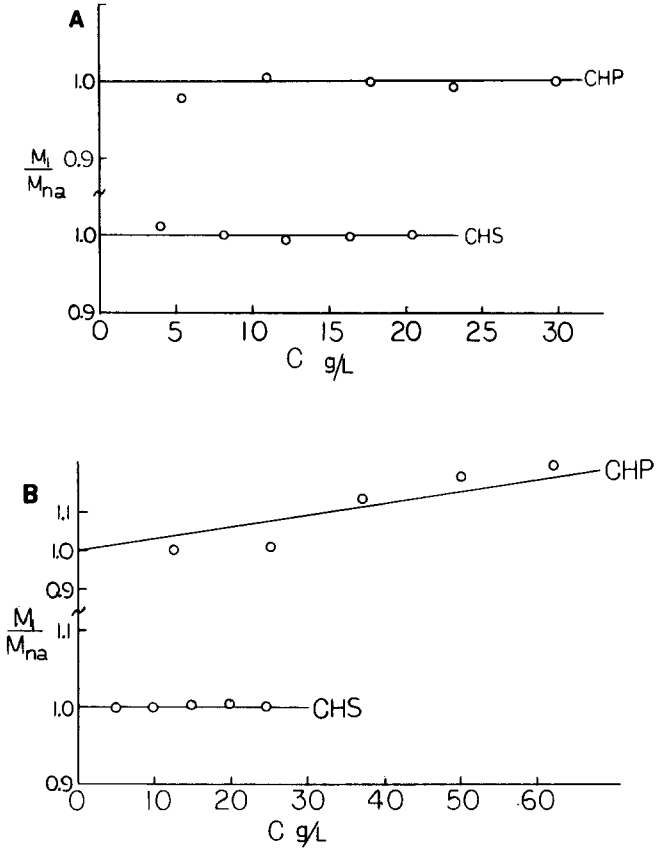

Fig. 5. Vapor pressure osmometry. Plot of E/c versus c for cholesteryl stearate (CHS) and cholesteryl palmitate (CHP) at 37°C in (A) $CHCl_3$ solutions and (B) CCl_4 solutions. Note that these esters do not self-associate in the solvents, whereas cholesterol does self-associate.

HETEROGENEOUS, NONASSOCIATING SOLUTES (1–3)

When the solute is heterogeneous and nonassociating, the osmotic pressure equation becomes

$$\frac{\Pi}{RT} = \frac{c}{M_{na}} = \frac{c}{M_n} + \frac{B_{OS}c}{2} \qquad Eq.\ 16)$$

Here B_{OS} is the osmotic pressure second virial coefficient; it is an indicator of the nonideal behavior of the solution. For an ideal solution $B_{OS} = 0$. The quantity M_n is the number average molecular weight, which is defined by

$$M_n = \frac{\sum_i n_i M_i}{\sum_i n_i} = c / \sum_i c_i / M_i \qquad Eq.\ 17)$$

Here n_i is the number of moles of the polymeric component i having a molecular weight M_i and a concentration c_i (in g/l), and c is the total solute concentration. The quantity M_{na} is the apparent number average molecular weight; eq. *16* can be recast as

$$\frac{1}{M_{na}} = \frac{1}{M_n} + \frac{B_{OS}c}{2}. \qquad Eq.\ 18)$$

Fig. 6 shows plots of Π/c versus c for aqueous dextran solutions (Pharmacia Dextran T-70, Lot 693) at various temperatures. This dextran has a molecular weight distribution that is similar to dextrans that are used as blood plasma extenders. The inclined straight lines indicate that the solution is nonideal; note how the nonideal effect (the slope of the plot) varies with temperature. For this sample Wan and Adams (17) obtained $M_n = (4.08 \pm 0.11) \times 10^4$. The values of M_n and B_{OS} are in **Table 2,** which also includes results at 20°C for a different dextran T-70 sample. Although, the dextran is a polysaccharide and not a lipoprotein or apolipoprotein, it illustrates how one can obtain M_n for a heterogeneous, nonassociating solute.

Heterogeneous, nonassociating solutes can also be studied by VPO; M_{na} (defined by eq. *18*) is obtained from

$$E/c = K_{VP}/M_{na} \qquad Eq.\ 5)$$

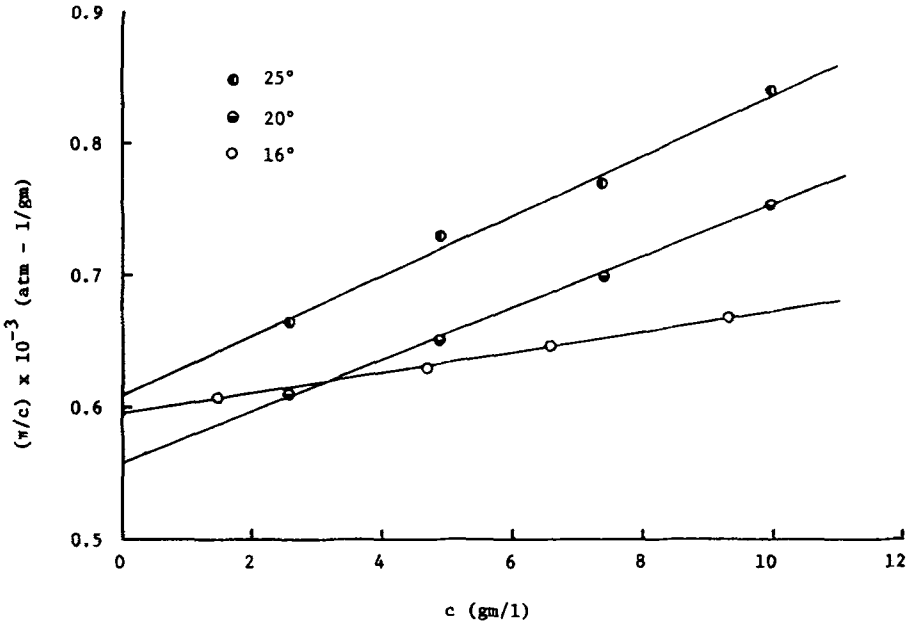

Fig. 6. Membrane osmometry. Plots of Π/c versus c for aqueous Dextran T-70 (Lot 693) solutions. This sample is heterogeneous and nonassociating. It forms nonideal solutions at the three temperatures. Note that the nonideal effect decreases with decreasing temperature. Reprinted with permission of Elsevier Science Publishers, ref. 16, Fig. 1.

where E, c, and K_{VP} are defined as before. Triton WR-1339 has been shown by Yamamoto et al. (18) to interact with plasma lipoprotein; they reported that it can displace apolipoprotein A-1 from the high density lipoprotein surface without the loss of lipid. They estimated the number average molecular weight of the unimer to be 4500 g/mol based on high performance liquid chromatography retention times (since Triton WR-1339 is heterogeneous to begin with, the number average molecular weight at infinite dilution will be that of the unimer; if the sample were homogeneous then one would use the term monomer). The same Triton WR-1339 sample was studied by VPO in $CHCl_3$ solutions at 37°C and at 45°C. **Fig. 7** shows plots of E/c versus c for these experiments; it is evident from these plots that Triton WR-1339 forms nonideal solutions with no self-association occurring. From these plots we obtained a value of $M_n = 2.806 \times 10^3$ g/mol at 37°C and 2.834×10^3 g/mol at 45°C, giving an average value of $M_n = (2.820 \pm 0.014) \times 10^3$ g/mol. The VPO measurements should be the more reliable ones.

TABLE 2. Values of M_n and B_{OS} at various temperatures (T)[a]

Lot number[b]	T (°C)	$M_n \times 10^{-4}$ (g/mol)	$B_{OS} \times 10^6$ ($l \cdot mol \cdot g^{-2}$)
7981	20	4.30	0.354
693	16	3.98	0.326
693	20.1	4.24	0.713
693	25	4.01	0.934

Average M_n $(4.08 \pm 0.11) \times 10^4$ for Lot 693

[a] These data are taken from Table 3 in reference 17; reprinted by permission of Elsevier Science Publishers, B.V.

[b] In a leaflet supplied with each Dextran T-70 sample, Pharmacia reported M_n by end group analysis. For Lot #693, $M_n = 3.95 \times 10^4$ g/mol and for Lot #7981, $M_n = 4.10 \times 10^4$ g/mol.

SELF-ASSOCIATIONS (3, 18)

Reversible chemical equilibria of the types

$$nP_1 \rightleftarrows P_n, \quad n = 2, 3, \cdots \qquad Eq.\ 19)$$

$$nP_1 \rightleftarrows qP_2 + mP_3 + \cdots \qquad Eq.\ 20)$$

and related equilibria are known as self-associations. Here P represents the self-associating solute. Self-associations may be discrete such as those described by eq. *19* or by an association similar to that shown in eq. *20*, but which has a discrete set of terms, such as a monomer–dimer–n-mer association. Sometimes self-associations appear to continue without limit; these are known as indefinite self-association (see eq. *20*). Self-associations are widely encountered; many proteins, such as lysozyme, chymotrypsin, apolipoproteins, β-lactoglobulins, have been reported to undergo self-association (19). Smaller molecules, such as ATP (20) (adenosine triphosphate), purine, and various other nitrogen bases and nucleosides (3, 21), cholesterol (11), bile salts (4), bile acid methyl esters (10), and methylene blue (22), also undergo self-associations under suitable solution conditions. **Fig. 8** shows plots of the osmotic

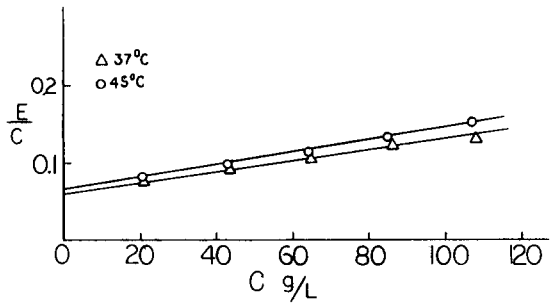

Fig. 7. Vapor pressure osmometry of Triton WR-1339 in $CHCl_3$ solutions at 37°C and at 45°C. These plots of E/c versus c indicate that the Triton WR-1339 behaves as a heterogeneous, nonassociating solute at both temperatures and forms a nonideal solution.

coefficient g, which is the same as M_1/M_{na}, versus total solute concentration c for cholesterol in $CHCl_3$ and CCl_4 solutions (11). Cholesterol self-associates in both solvents, but it is clear from this figure that the solvent certainly influences the self-association.

In order to analyze self-associations, including the nonideal case, one assumes that the natural logarithm of the activity coefficient y_i of self-associating species i obeys the relationship (3, 23)

$$\ln y_i = iB^* M_1 c, \quad i = 1, 2, \cdots \qquad Eq.\ 21)$$

Here M_1 is the molecular weight of the monomer and B* is a constant whose value depends on the solute–solvent combination and the temperature. When eq. *21* is obeyed, it follows that the ratio

$$y_n/y_1^n = 1 \qquad Eq.\ 22)$$

and that the total solute concentration is

$$c = c_1 + k_2 c_1^2 + k_3 c_1^3 + \cdots \qquad Eq.\ 23)$$

For self-associations it has been shown that one can obtain M_{na}, the apparent number average molecular weight, from membrane osmometry by (3, 23)

$$\Pi/RT = c/M_{na} \qquad Eq.\ 24)$$

and from vapor pressure osmometry by (3, 9, 11)

$$E/K_{vp} = c/M_{na}. \qquad Eq.\ 25)$$

The osmotic coefficient, g, is given by

$$g = M_1/M_{na} = \frac{M_1}{M_{nc}} + \frac{BM_1 c}{2} \qquad Eq.\ 26)$$

where $B = B^* + (\bar{v}/1000\ M_1)$. The quantity B is the nonideal term, and the subscript M_{nc} indicates that the number average molecular weight (and also other average molecular weights) are concentration-dependent because of the self-association. Note that $\lim_{c \to 0} M_{nc} = M_1$, the monomer molecular weight. For self-associations $M_q = qM_1$, where q = 2, 3, ... The concentration dependence of M_{nc} is evident from

$$cM_1/M_{nc} = \sum_i c_i/i = c_1 + \frac{k_2 c_1^2}{2} + \frac{k_3 c_1^3}{3} + \cdots \qquad Eq.\ 27)$$

Here c_i represents the concentration of species i, i = 1, 2, ... Analogous equations are available for other average molecular weights. Adams et al. (3), Robeson et al. (10), and Foster et al. (11) have shown that the apparent values of the weight average molecular weight (M_{wa}) and the weight fraction of monomer (f_a) can be obtained from M_{na} versus c values, since

$$\frac{M_1}{M_{wa}} = \frac{d}{dc}\left(\frac{cM_1}{M_{na}}\right) = \frac{M_1}{M_{na}} + c\frac{d}{dc}\left(\frac{M_1}{M_{na}}\right)$$

$$= \frac{M_1}{M_{wa}} + BM_1 c \qquad Eq.\ 28)$$

and

$$\ln f_a = \int_0^c \left(\frac{M_1}{M_{na}} - 1\right)\frac{dc}{c} + \frac{M_1}{M_{na}} - 1$$

$$= \ln f_1 + BM_1 c. \qquad Eq.\ 29)$$

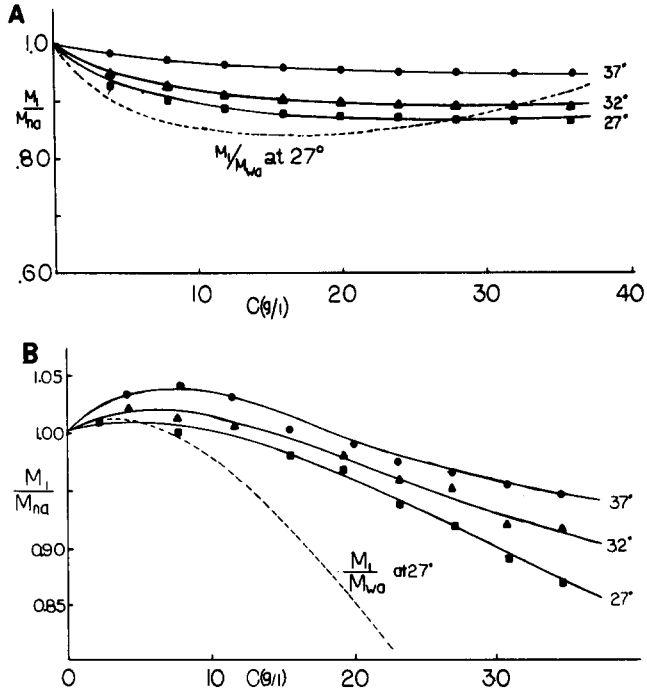

Fig. 8. Self-association of cholesterol. Shown are plots of the osmotic coefficient of ($g = M_1/M_{na}$) versus total solute concentration c at various temperatures for cholesterol solutions in (A) $CHCl_3$ and (B) CCl_4. Cholesterol self-associates in both solvents, and the self-association is clearly temperature-dependent. Notice the difference in the two sets of plots. The dashed lines indicate values of M_1/M_{wa} that were obtained from eq. *28*. $CHCl_3$ can form weak hydrogen bonds with cholesterol; CCl_4 cannot form hydrogen bonds with cholesterol. These data were obtained by vapor pressure osmometry. Reprinted with the permission of the American Chemical Society, ref. 11, Figs. 1 and 2.

These values of M_{na}, M_{wa}, and ln f_a can be evaluated without prior knowledge of the type of self-association present, and they can be combined in various ways to test for the type of self-association present and also be used in the subsequent evaluation of the equilibrium constant(s) k_i and the nonideal term BM_1. Two useful functions are (3, 10, 11)

$$\xi = \frac{2M_1}{M_{na}} - \frac{M_1}{M_{wa}} = \frac{2M_1}{M_{nc}} - \frac{M_1}{M_{wc}} \qquad Eq.\ 30)$$

and

$$\nu = \frac{2M_1}{M_{na}} - \ln f_a = \frac{2M_1}{M_{nc}} - \ln f_1. \qquad Eq.\ 31)$$

If the self-association were a monomer–n-mer association (see eq. *10*), then it can be shown that

$$\xi = \frac{2 + 2(n-1)f_1}{n} - \frac{1}{n-(n-1)f_1} \qquad Eq.\ 32)$$

or

$$\nu = \frac{2 + 2(n-1)f_1}{n} - \ln f_1 \quad (0 \le f_1 \le 1) \qquad Eq.\ 33)$$

Equation *32* can be recast as a quadratic equation in ξ, and eq. *33* can be solved by successive approximations. Since

$$f_1 = c_1/c \qquad Eq.\ 34a)$$

$$f_n = k_n c_1^{\,n}/c \qquad Eq.\ 34b)$$

and

$$f_n = 1 - f_1 \qquad Eq.\ 34c)$$

for a monomer–n-mer association, one can obtain k_n from a plot of $(1 - f_1)/f_1^{\,n}$ versus c^{n-1} since

$$(1 - f_1)/f_1^{\,n} = k_n c_1^{\,n-1} \qquad Eq.\ 35)$$

When n is not known ahead of time, one must make estimates of it (n = 2, 3, · · ·), insert these estimates in the equation for ξ or ν, and evaluate f_1. For the correct model, a plot of $(1 - f_1)/f_1^{\,n}$ versus c^{n-1} will give a straight line going through or close to the origin with a slope of k_n. If the model is wrong, then one may get imaginary values of f_1 from eq. *32*, the plot based on eq. *35* may be curved, or negative values of k_n (which is physically impossible) may be obtained. Other procedures involving ν, ξ, or η (here $\eta = \frac{M_1}{M_{wa}} - \ln f_a = \frac{M_1}{M_{wc}} - \ln f_1$) can be used to test and analyze for other self-associations; these procedures are described extensively elsewhere (3, 10, 11, 24).

Fig. 8 shows plots of g (g = M_1/M_{na}) versus c for cholesterol in $CHCl_3$ and CCl_4 solutions at different temperatures (11). Note that these self-associations are temperature-dependent; the extent of self-association increases as the temperature decreases. The self-association is quite different in the two solvents. This solvent effect may be due to the fact that $CHCl_3$ is a hydrogen-bonding solvent, whereas CCl_4 is not. In $CHCl_3$ the self-association could be described as a monomer–dimer association or as an indefinite self-association in which all odd species (trimer, etc.) other than monomer are absent and which requires only one equilibrium constant. This model is known as a Type II SEK (sequential, equal equilibrium constant) association.[2] It is not unusual for weak associations to be described by more than one model (24). In CCl_4 the self-association was clearly nonideal; this is evident from the maximum in the plot of g versus c near zero solute concentration which is followed by the decrease in g with increasing c. This association was described as a monomer–trimer–hexamer (1–3–6) association. Note that the cholesteryl esters do not exhibit self-association in these solvents (see Figs. 5A and 5B).

It is possible to analyze three component aqueous solutions containing ionizable solutes by two routes. Bile salts are natural surfactants that aid in the solubilization of lipids. In aqueous solution they ionize and also self-associate. In order to study their self-associations, one must use a supporting electrolyte, like NaCl, to overcome the primary charge effect. **Fig. 9** shows a plot of g versus c at 37°C for the bile salt, sodium cholate, in isotonic saline solutions (4). Here the solutions were carefully dialyzed, in a hollow fiber dialyzer with a 200-g cut-off, against isotonic saline. The curve shown in Fig. 9 could be analyzed as a Type IV SEK indefinite self-association. This is a cooperative variant of the Type II SEK association in which all odd species beyond the monomer (trimer, etc.) are absent but all even species (dimer, tetramer, etc.) are present. It can be described by one nonideal term and two equilibrium constants: k_{12} for the monomer–dimer equilibrium and k for all other associations of the form $P_2 + P_{n-2} \rightleftarrows P_n$ (n = 4, 6, 8, · · ·). One can also use partition isotherms together with vapor pressure osmometry to study self-associations. Vadnere and Lindenbaum (25) studied the self-association of bile salts by partitioning radioactively labeled bile salts between an aqueous Tris buffer solution and 1-octanol. Since the bile salt undergoes hydrolysis, it is the bile acid that is soluble in the 1-octanol. The ratio of the total concentration of solute in each phase is the apparent partition (or distribution) coefficient (K_{app}); the $\lim_{c \to o} K_{app} = K_{PAR}$, the partition coefficient. Adams and Beckerdite (26) have shown that $Q = K_{app}/K_{PAR} = f_a^{(AQ)}/f_a^{(OR)}$. Here $f_a^{\,i}$ is the weight fraction of monomer in phase i (i = aqueous (Aq) or organic (Or)). When one

[2] There are two general types of indefinite self-associations: the SEK model and the AK (attenuated equilibrium constant) model. A brief description of these models is in the appendix.

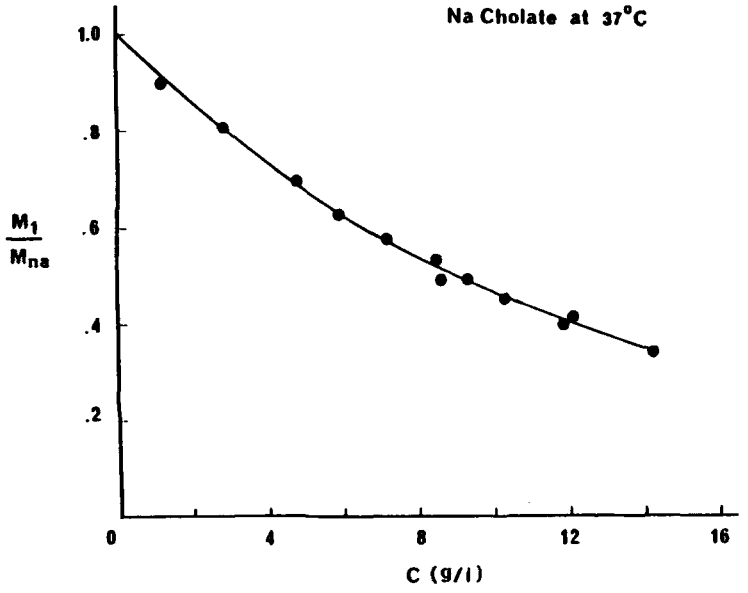

Fig. 9. Self-association of sodium cholate in 0.154 M NaCl at 37°C. Plots of the osmotic coefficient g ($g = M_1/M_{na}$) versus total solute concentration c. This self-association could be described by a two-equilibrium-constant model, a Type IV SEK indefinite self-association. Reprinted by permission of Elsevier Science Publishers, ref. 4, Fig. 4.

does vapor pressure osmometry experiments on the organic phase, then one can establish the state of aggregation in the organic phase and evaluate $f_a^{(OR)}$. Then one knows $f_a^{(AQ)}$ which is related to M_{na} (and M_{wa} if the solute self-associates). Thus from these studies one can analyze self-associations in either or both phases and evaluate the equilibrium constant or constants and the nonideal term, and in addition one can evaluate the partition coefficient (K_{PAR}). When temperature-dependent studies are done, one can obtain the thermodynamic functions ($\Delta G°$, $\Delta H°$, and $\Delta S°$) for self-association and also for transfer between phases. At present, this procedure appears to be restricted to small molecules, since most proteins are not soluble in organic solvents.

At present, I am not aware of any studies on the self-association of lipoproteins or apolipoproteins by membrane osmometry. Two proteins exhibiting self-association, which have been studied by membrane osmometry, are chicken erythrocyte histone F2b (1) and Bowman-Birk soybean trypsin inhibitor (27). Both proteins have been studied under various solution conditions, and the changes in the solution conditions do influence the observed self-association.

MIXED ASSOCIATIONS (3)

Associations of the type

$$A + B \rightleftarrows AB, \qquad Eq.\ 36)$$

or

$$nA \rightleftarrows A_n$$

$$A + B \rightleftarrows AB \qquad Eq.\ 37)$$

and related associations involving two different reactants, A and B, are known as mixed associations. According to Steiner (28) mixed associations are frequently encountered in biochemistry, but are rarely encountered in print. The reason for this is that, first of all, each reactant must be studied separately to determine its state of aggregation. When no association is present, the molecular weight (M_i) and the nonideal term (B_{ii}) must be determined. When self-association occurs, the type self-association present as well as the equilibrium constant or constants (k_i) and the nonideal term (B_{ii}) must be determined. Then one can study the mixed association. Note that at a fixed total concentration (e.g., 10 mg/ml or 10 g/l) there are a vast number of ways one can blend A and B (even when no mixed association occurs) to get the same total concentration; for each blend there would be a different value of M_n, the number average molecular weight. In order to study mixed associations, one first prepares separate solutions of A and B, dialyzing them in the same buffer when one is dealing with proteins. Then one makes a working stock solution having a fixed molar ratio of A and B (or a fixed weight ratio of A and B), assuming no mixed association occurs. The symbol $\beta_m = M_B°/M_A°$ indicates a fixed molar ratio of A and B, whereas $\beta_g = c_B°/c_A°$ indicates the fixed weight ratio of A and B. A series

of dilutions at constant β_m or β_g are prepared, and membrane or vapor pressure osmometric experiments are performed on each solution. Usually, two or more series of experiments at different β_g (or β_m) must be performed. Then one can perform the analysis.

Suppose the mixed association were described by eq. *36*, then for the ideal case one would have the following information available (3):

$$\beta g = c_B^\circ/c_A^\circ \quad \text{or} \quad \beta_m = m_A^\circ/m_B^\circ. \qquad \textit{Eq. 38)}$$

The mass balance equation for each reactant is

$$c_A^\circ = c_A + (kc_Ac_B/M_{AB})M_A \qquad \textit{Eq. 39a)}$$

$$c_B^\circ = c_B + (kc_Ac_B/M_{AB})M_B. \qquad \textit{Eq. 39b)}$$

The total solute concentration is

$$c = c_A^\circ + c_B^\circ = c_A + c_B + kc_Ac_B. \qquad \textit{Eq. 40)}$$

The stoichiometric number of moles is

$$\frac{c}{M_n^\circ} = \frac{c_A^\circ}{M_A} + \frac{c_B^\circ}{M_B} = \frac{c_A}{M_A} + \frac{c_B}{M_B} + \frac{2kc_Ac_B}{M_{AB}}. \qquad \textit{Eq. 41)}$$

The equilibrium number of moles is

$$\frac{\Pi}{RT} = \frac{c}{M_n^{eq}} = \frac{c_A}{M_A} + \frac{c_B}{M_B} + \frac{kc_Ac_B}{M_{AB}}. \qquad \textit{Eq. 42)}$$

Here M_n° is the number average molecular weight for a nonassociating blend of A and B; M_n^{eq} is the number average molecular weight of the blend when a mixed association is present. When eq. *42* is subtracted from eq. *41*, the result is

$$\Delta(c/M_n) = \frac{c}{M_n^\circ} - \frac{c}{M_n^{eq}} = \frac{kc_Ac_B}{M_{AB}} \qquad \textit{Eq. 43)}$$

for the mixed association described by eq. *36*. One can rearrange the mass balance equations (eqs. *39a* and *39b*) to obtain

$$c_A = c_A^\circ - (kc_Ac_B/M_{AB})M_A = c_A^\circ - (\Delta c/M_n)M_A \qquad \textit{Eq. 44a)}$$

$$c_B = c_B^\circ - (kc_Ac_B/M_{AB})M_B = c_B^\circ - (\Delta c/M_n)M_B. \qquad \textit{Eq. 44b)}$$

Insertion of these equations into eq. *43* leads to

$$\Delta(c/M_n) = \frac{k}{M_{AB}}[c_A^\circ - (\Delta c/M_n)M_A][c_B^\circ - (\Delta c/M_n)M_B]. \qquad \textit{Eq. 45)}$$

Thus a plot of $\Delta(c/M_n)$ versus the product on the right hand side of eq. *45* will be a straight line going through the origin with a slope of k/M_{AB}. Experimental error could cause the line to go close to, but not through, the origin. Upward curvature of such a plot suggests higher associated species might be present, and nonideal effects could cause downward curvature. Similar plots can be developed for more complex mixed associations; for more details see ref. (3).

Steiner (29) has developed some elegant methods for evaluating the number fraction of each reactant under ideal conditions for a variety of mixed associations; his theory can be extended to the nonideal case (28). For the ideal case, Steiner (29) showed that

$$\ln x_A + \beta_m \ln x_B = \int_0^m (g-1)\frac{dm}{m} + \ln\frac{1}{1+\beta_m} + \beta_m \ln\frac{\beta_m}{1+\beta_m} = \Phi. \qquad \textit{Eq. 46)}$$

Here $g = M_n^\circ/M_n^{eq}$ is the osmotic coefficient, $m = c/M_n^{eq}$ is the equilibrium molarity, $X_A = m_A/m = (c_A/M_A)/(c/M_n^{eq})$ is the number fraction of A, and $X_B = m_B/m = (c_B/M_B)/(c/M_n^{eq})$ is the number fraction of B. For a mixed association described by eq. *36* with m = 1, one notes that $c_A = c_B$ and $X_A = X_B$, thus

$$\ln x_A = \frac{1}{2}\int_0^m (g-1)\frac{dm}{m} + \ln(1/2). \qquad \textit{Eq. 47)}$$

Other mixed associations are more complicated to analyze.

We have studied the interaction of methyl lithocholate (MeLC) and also methyl cholate (MeC) with cholesterol (CH) in $CHCl_3$ solutions at 37°C (30). **Fig. 10** shows the results with MeLC. In $CHCl_3$ solutions, an ideal solution is formed from two different blends of MeLC with CH; for this case the osmotic coefficient g = 1. This figure also shows the plots of the osmotic coefficient, g versus c for both MeLC (no self-association) and cholesterol (a weak self-association). **Fig. 11** shows plots of g versus c for cholesterol, MeC, and for three different blends of CH and MeC in $CHCl_3$ at 37°C. Both reactants, cholesterol and methylcholate, undergo self-association, as is evident from the plots of g versus c for cholesterol and for MeC. The blends of cholesterol and MeC also undergo associations, as is evident from the g versus c plots. The analysis of the data indicated that methyl cholate and cholesterol underwent a nonideal A + B ⇄ AB mixed association and both reactants exhibited self-associations.

DISCUSSION

It is evident from the preceding material that membrane and vapor pressure osmometry can be used to obtain molecular weights or number average molecular weights or their apparent values under nonideal conditions. These techniques can also be used to study self-associations or mixed associations. Theoretically, the osmometric methods are less complicated than are the theories for sedimentation equilibrium or elastic light

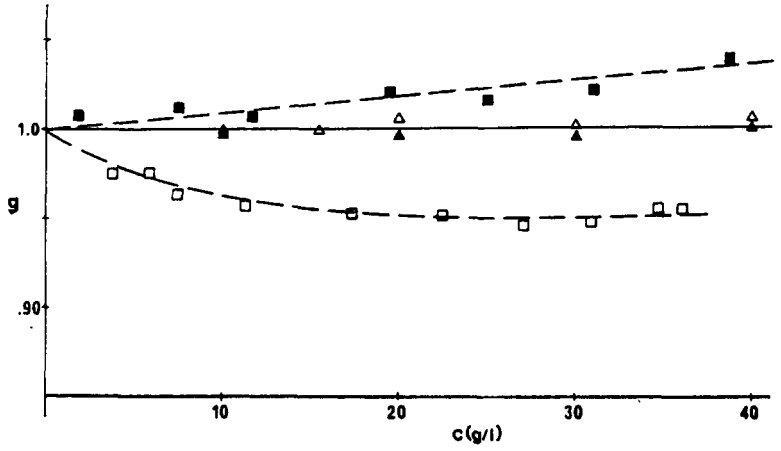

Fig. 10. Mixtures of methyl lithocholate and cholesterol in $CHCl_3$ solutions at 37°C. This figure shows plots of the osmotic coefficient g versus total concentration c for methyl lithocholate (top plot), two different blends of cholesterol and methyl lithocholate (middle plot), and for cholesterol (bottom plot). Note that the plot of g versus c for methyl lithocholate (top) indicates no self-association and that the solution is slightly nonideal. The bottom plot indicates that cholesterol does undergo self-association. The middle plot indicates that both mixtures of cholesterol and methyl lithocholate form ideal solutions, since g = 1. This would indicate that there is no mixed association between cholesterol and methyl lithocholate. Reprinted with permission from Elsevier Science Publishers, ref. 28, Fig. 2.

scattering. One does not need to determine the refractive index increment ψ; this is necessary for light scattering and sedimentation equilibrium experiments that use refractometric optics. It is not necessary to determine the partial specific volume $\bar{v}$ or the density increment $(\partial\rho/\partial c)_{T,\mu}$; these quantities are needed in sedimentation equilibrium experiments. In studying mixed associations by sedimentation equilibrium or light scattering experiments, one may have to make assumptions about the values of ψ, $\bar{v}$, or $(\partial\rho/\partial c)_{T,\mu}$, for the complex or complexes (AB, A_2B, etc.) that are formed (31); this is unnecessary in osmometry. In principle, elastic light scattering should be the fastest way to obtain M, M_w, M_{app}, or M_{wapp}, as well as the fastest way to study self- and mixed associations. In practice it is very difficult to remove dust from aqueous solutions. That dust causes problems in light scattering is evident from problems in well-known textbooks (see p. 504 in ref. 7 or p. 232 in ref. 8). One quickly discovers that a very slight contamination by dust can cause a severe error in light scattering experiments.

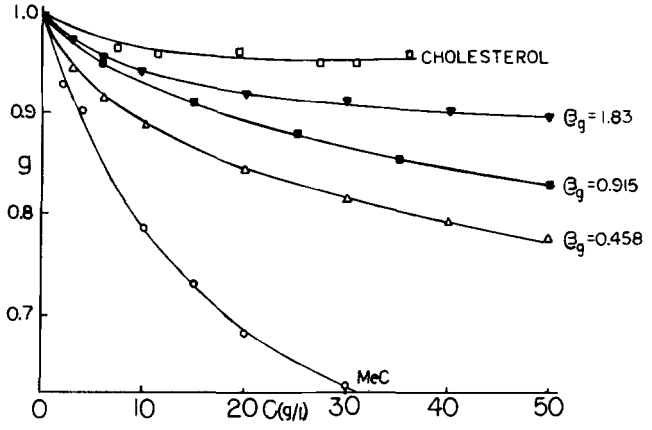

Fig. 11. Mixed association of cholesterol and methyl cholate in $CHCl_3$ solutions at 37°C. The plots of g versus c for the top plot (cholesterol) and the bottom plot (methyl cholate) indicate that these solutes undergo self-association in $CHCl_3$ solutions at 37°C. Note how much stronger the self-association of methyl cholate is; this association could be described as monomer–dimer–trimer association. A comparison of the plots of g versus c for methyl lithocholate (see top plot in Fig. 10) and for methyl cholate indicates that increasing the number of hydroxyl groups increases the likelihood of self-association. The three blends of cholesterol and methyl lithocholate (the three middle plots) indicate that a mixed association is present here, whereas no mixed association occurs with the cholesterol–methyl lithocholate mixtures. The mixed association between cholesterol and methyl cholate could be described as one in which an A + B ⇄ AB complex was formed and in which both reactants underwent self-association. Reprinted with permission from Elsevier Science Publishers, ref. 28, Fig. 3.

Osmometric techniques can be used over a wide temperature range: from −5°C (for the Wescan) or ambient temperature (Knauer) to 130°C. Unless the ultracentrifuge is equipped for high temperature operation it is difficult to do experiments above 30°C because drive oil fogs the schlieren lenses. Osmometric techniques are relatively fast and nondestructive; the sample can be recovered, if desired. Membrane osmometry could be used along with other physical techniques, such as optical rotary dispersion (ORD) or circular dichroism (CD), to correlate changes in conformation with changes in the state of aggregation or in ideal/nonideal behavior. For instance, a comparison of Figs. 1 and 2 in the paper by Castellino and Barker (32) indicates that native proteins may exhibit

quasi-ideal behavior that is changed on denaturation. One could follow changes in the ORD or CD with changes in the molecular weight and the osmotic pressure second virial coefficient. It should be possible to study the effect of promoters and inhibitors on self-associating solutes. Careful experiments could indicate how much of a promoter is needed to initiate self-association or how much of an inhibitor is needed to partially or completely stop self-association. The effect of various cofactors or adjuvants on apoenzymes could be monitored by following changes in the state of aggregation. With methyl cholate solutions in $CHCl_3$, ^{13}C spin lattice relaxation times obtained from nuclear magnetic resonance experiments indicated which parts of the aggregate were tightly bound and which were less tightly bound (10). The effects of changes in solvents (or buffers) and in chemical composition on self-associations or mixed associations can be studied by osmometry. It is evident from Fig. 10 that methyl lithocholate (MeLC) does not self-associate, and that MeLC and cholesterol do form an ideal solution in $CHCl_3$ at 37°C. On the other hand, from Fig. 11 one notes that methyl cholate (MeC) does self-associate and that MeC and cholesterol do undergo a mixed association. The data analysis indicates that an AB complex is formed.

Molecular weights obtained by membrane osmometry (or by vapor pressure osmometry) show good agreement with molecular weights obtained by other methods (33). Note the good agreement between the sedimentation equilibrium and membrane osmometry values for the molecular weight of canine high density lipoprotein. Small amounts of material are sufficient for membrane or vapor pressure osmometry. A reduction insert is available for the Knauer membrane osmometer; with this insert one needs only 50 μl per individual rinse or measurement.

Two commercial membrane osmometers are available; the Wescan (formerly Melabs), available from Wescan Instruments, Santa Clara, CA, and the Knauer, available from UIC, Joliet, IL. For aqueous solutions, one can use cellulose acetate membranes (3) (available from Schleicher and Schuell, Keene, NH), Amicon YM-10 or PM-10 membranes, or ATF (asymmetric thin film) membranes, available from Wescon Instruments. Although one could use dialysis membranes in the osmometer, these may be more retentive so that longer time periods are required to reach osmotic equilibrium. Cellulose membranes (3) are usually used in nonaqueous solvents. Generally, membrane osmometry is preferred for molecules having a molecular weight range of 10,000 to 1,000,000. The sensitivity of the osmometry decreases as the molecular weight increases (see eq. *14*). One cannot study micelles by membrane osmometry, since the monomers are of lower molecular weight and can diffuse through the membrane. Both osmometers can be used over a range of temperatures, but the Wescan, which has a solid-state cooler can be operated at lower temperatures. Osmometry is an absolute method, i.e., molecular weights can be obtained directly from the data. Both osmometers require a calibration step; here one adjusts the chart recorder so that a change of 10 cm in the hydrostatic pressure in the osmometers corresponds to a full-scale deflection of the chart recorder.

Two vapor pressure osmometers are available: the Knauer and the Corona-Wescon. A universal probe is available that allows one to do experiments with either aqueous or nonaqueous solutions. Both instruments can be operated over a wide range of temperatures, from ambient temperature (ca. 25°C) to 130°C.

For directions on performing vapor pressure osmometry experiments or membrane osmometry experiments, one should consult the appropriate manuals for these instruments or consult ref. (3). One important precaution in membrane osmometry is to avoid bubbles. The buffer or solvent and the membranes should be degassed prior to using them in the osmometer. One should avoid trapping bubbles under the membrane when installing it, and one should degas each solution before starting measurements on it.

APPENDIX

Osmotic pressure equation (1–3)

The chemical potential of the solvent, μ_0, can be represented by

$$\mu_0 = \mu_0^\bullet + RT \ln a_0 = \mu_0^\bullet + RT \ln \gamma_0 x_0 \qquad Eq.\ A1)$$

or by

$$\mu_0 = \mu_0^\bullet + gRT \ln x_0. \qquad Eq.\ A2)$$

Here μ_0 is the chemical potential of the solvent, $\mu_0^\bullet$ is the value of μ_0 in the standard state (which is the pure solvent at temperature T), R is the gas constant, a_0 is the activity of the solvent whose mole fraction is x_0 and whose activity coefficient is γ_0, and g is the osmotic coefficient of the solvent. At constant temperature T, when the solution is ideal $\gamma_0 = 1$, $g = 1$, and $a_0 = x_0$. In Fig. 1 when the pressure on side 2 is increased from P to $P + \Pi$, the solvent chemical potential (in side 2) changes from $\mu_0(T, P, x_1)$ to $\mu_0(T, P + \Pi, x_1)$. Note that the increased pressure raises the chemical potential of the solvent on side 2. This can be represented by

$$\mu_0(T, P + \Pi, x_1) - \mu_0(T, P, x_1) = \int_P^{P+\Pi} (\partial\mu/\partial P)_{T,n}\, dP \cong \Pi \bar{V}_0. \qquad Eq.\ A3)$$

Here

$$(\partial\mu_0/\partial P)_{T,n} = \bar{V}_0 \qquad Eq.\ A4)$$

where $\bar{V}_0$ is the partial molar volume of the solvent. It is generally assumed that $\bar{V}_0$ is independent of pressure for small pressure changes; the pressure changes usually encountered in osmotic pressure experiments are less than one atmosphere. Also note that since there is no flow of solvent across the semipermeable membrane at osmotic equilibrium, the chemical potential of the solvent must be the same on both sides. Therefore,

$$\mu_0(T, P + \Pi, x_1) = \mu_0^\bullet \qquad Eq.\ A5)$$

From eqs. *1* and *5* it follows that

$$\mu_0(T, P + \Pi, x_1) - \mu_0(T, P, x_1) = -RT \ln a_1. \qquad Eq. A6)$$

Consequently, from eqs. *3* and *6* one obtains

$$\Pi \bar{V}_0/RT = -\ln a_0. \qquad Eq. A7)$$

Alternatively, one notes from eqs. *2* and *5* that

$$\mu_0(T, P + \Pi, x_1) - \mu_0(T, P, x_1) = -gRT \ln x_0. \qquad Eq. A8)$$

From eqs. *3* and *8* one obtains

$$\Pi \bar{V}_0/RT = -g \ln x_0. \qquad Eq. A9)$$

Equations *7* and *9* form the basis for obtaining the working osmotic pressure equations.

Alternatively, one can develop the osmotic pressure equation, eq. *14*, from the following relations:

$$V dP = n_1 d\mu_1 \qquad Eq. A10)$$

$$\mu_1 = \mu_1^0 + RT \ln a_1 = \mu_1^0 + RT \ln y_1 c \qquad Eq. A11)$$

$$\ln y_1 = B * Mc + \cdots \qquad Eq. A12)$$

and

$$\left(\frac{\partial \mu_1}{\partial P}\right)_{T,n_0} = \bar{V}_1 = M_1 \bar{v}_1 \qquad Eq. A13)$$

Here, n_1 is the number of moles of solute, whose chemical potential is μ_1 and whose activity is a_1. The solute activity $a_1 = y_1 c$, where y_1 is the activity coefficient of solute whose concentration is c. The quantity μ_1^0 is the standard state chemical potential of the solute. In eq. *A12*, $B*$ is a nonideal term whose value depends on the temperature and on the solute–solvent combination. $\bar{V}_1$ is the partial molar volume of the solute, and $\bar{v}_1$ is the partial specific volume of the solute. The osmotic pressure second virial coefficient B in eqs. *14* or *15* is defined by

$$B = B* + \frac{\bar{v}_1}{1000M}. \qquad Eq. A14)$$

Vapor pressure osmometry equation (3, 9)

Whenever a solution is made up, the vapor pressure of the pure solvent (p_0^*) is lowered, and the relative vapor pressure lowering ($\Delta p/p_0^*$) is related to the mole fraction of solute (x_1) by Raoult's law

$$\Delta p/p_0^* = gx_1 \qquad Eq. B1)$$

where g is the osmotic coefficient. For an ideal solution g = 1. Whenever liquid and vapor are in equilibrium, the Clausius-Clapeyron equation applies for changes in temperature and pressure. The condensation of solvent on the solution thermistor (B in Fig. 2) causes a slight change in temperature. Thus

$$\ln (p_0/p_0^*) = \frac{-\Delta H_{vap}}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \cong \frac{-(\Delta H_{vap})\Delta T}{RT_1^2} \qquad Eq. B2)$$

where $\Delta T = T_1 - T_2$ and T_2 is very close to T_1. Now note that

$$\ln (p_0/p_0^*) = \ln\left(\frac{p_0 - p_0^* + p_0^*}{p_0^*}\right) = \ln\left(1 - \frac{\Delta p}{p_0^*}\right) \cong -\frac{\Delta p}{p_0^*} \qquad Eq. B3)$$

Here $\Delta p = p_0^* - p_0$ is the vapor pressure lowering of the solvent. From eqs. *B1* to *B3* it follows that

$$\frac{\Delta p}{p_0^*} = gx_1 = \frac{(\Delta H_{vap})\Delta T}{RT_1^2} \qquad Eq. B4)$$

or

$$(\Delta T)_{Th} = \left(\frac{RT_1^2}{\Delta H_{vap}}\right) gx_1 = K_{Th} gx_1. \qquad Eq. B5)$$

The subscript Th indicates that this would be the case when equilibrium thermodynamics applies; it shows that the relative vapor pressure lowering is related to a temperature difference between the two thermistors in Fig. 2. Actually, vapor pressure osmometry is a steady state experiment in which radiative heat losses in the thermostatted chamber are balanced by condensation on the solution drop. Thus, the steady state temperature change, $(\Delta T)_{ss}$, is actually lower than the thermodynamic temperature change, $(\Delta T)_{Th}$. It has been shown that

$$(\Delta T)_{ss} = K_{ss} gx_1 \qquad Eq. B6)$$

where K_{ss} is another constant relating $(\Delta T)_{ss}$ to gx_1. The temperature difference $(\Delta T)_{ss}$ is usually measured as a resistance change (Δr) or as a microvolts imbalance (E) on the Wheatstone bridge circuit of the VPO. Since E (or Δr) is proportional to $(\Delta T)_{ss}$, one notes that (3)

$$E = K_{vp}(c/M_{na}) \qquad Eq. B7)$$

when the sample is heterogeneous and nonassociating. Here K_{vp} is an instrumental, operational constant whose value depends on the temperature and the solute–solvent combination, c is the total solute concentration, and M_{na} is the apparent number average molecular weight of the solute. K_{vp} is determined from VPO experiments under the same experimental conditions using a nonvolatile solute of known molecular weight. When the sample is homogeneous and nonassociating, one obtains

$$E = K_{vp}(c/M_{app}) \qquad Eq. B8)$$

where M_{app} is the apparent molecular weight of the solute.

Indefinite self-associations

These self-associations are described by eq. *20*. They can be considered to be made up of a series of successive chemical equilibria of the type

$$P_1 + P_1 \rightleftarrows P_2$$

$$P_1 + P_2 \rightleftarrows P_3$$

$$\vdots$$

$$P_1 + P_{i-1} \rightleftarrows P_i (i = 3, 4, \cdots). \qquad Eq. C1)$$

Garland and Christian (34) pointed out that indefinite self-associations can be divided into two groups. One group is known as the sequential, equal equilibrium constant (SEK) model and the other group is known as the attenuated equilibrium constant (AK) model. For the SEK Type I model, it is assumed that all molar equilibrium constants, K_i, for any step

$$P_1 + P_{i-1} \rightleftarrows P_i \quad (i = 2, 3, \cdots) \qquad Eq. C2)$$

$$K_i = \frac{[P_i]}{[P_1][P_{i-1}]} \qquad Eq. C3)$$

are the same. Here $[P_i]$ is the molar concentration of species i. This leads to the following relations on the g/l concentration scale, when $kc_1 < 1$:

$$c = c_1 + 2kc_1^2 + 3k^2c_1^3 + 4k^3c_1^4 + \cdots = c_1/(1 - kc_1)^2; \qquad Eq. C4)$$

when $kc_1 < 1$:

$$k = K/M_1 = \text{intrinsic equilibrium constant} \qquad Eq. C5)$$

$$\frac{M_1}{M_{nc}} = 1 - kc_1 \qquad Eq. C6)$$

$$\frac{M_1}{M_{wc}} = \frac{1 - kc_1}{1 + kc_1}. \qquad Eq. C7)$$

Usually the Type I SEK model can be described by one equilibrium constant (k) and a nonideal term. The Type II SEK model assumes that all odd species beyond monomer are absent. This can also be described by one equilibrium constant (k) and one nonideal term (BM_1). By analogy with binding studies, the SEK Type I and II models are known as noncooperative associations. The Type I SEK model is also

known as a random, open, or isodesmic association. Two other models for the SEK association assume that the molar equilibrium constant K_{12} or K_2 for $P_1 + P_1 \rightleftarrows P_2$ is different from the molar equilibrium constant K for the other steps, i.e., for $P_1 + P_{i-1} \rightleftarrows P_i$ ($i = 3, 4, \cdots$). The Type III SEK model and the Type IV SEK model are cooperative variants of the Types I and II SEK models, respectively. For details on how these associations might be analyzed, the reader should consult ref. 24.

In the AK indefinite self-association model Type I, it is assumed that all species are present, but the K_i in eq. *C2* becomes

$$K_i = K/i, \quad i = 2, 3, \cdots \qquad \textit{Eq. C8}$$

Here the integer i is the attenuation factor. For the Type I AK model, it is not required that $kc_1 < 1$; thus $0 < kc_1 < \infty$ for the AK models. For the Type I AK model one obtains:

$$c = c_1 + kc_1^2 + k^2c_1^2/2! + k^3c_1^3/3! + \cdots$$

$$= c_1 \exp(kc_1), \quad 0 < kc_1 < \infty \qquad \textit{Eq. C9}$$

$$\frac{M_1}{M_{nc}} = \frac{1}{kc_1}[1 - \exp(-kc_1)] \qquad \textit{Eq. C10}$$

$$\frac{M_1}{M_{wc}} = \frac{1}{1 + kc_1}. \qquad \textit{Eq. C11}$$

The Type II AK model is analogous to the Type II SEK model in that all odd species beyond the monomer are absent. The Types III and IV AK models are two-equilibrium constant variants, respectively, of the Type I and II AK models. In some cases it may be impossible to distinguish between comparable AK and SEK models; see refs. 24 and 34 for details.

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