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MIXED ASSOCIATION OF CHOLESTEROL WITH METHYL CHOLATE AND METHYL LITHOCHOLATE IN CHLOROFORM SOLUTIONS

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The concentration-dependent mixed association behavior of cholesterol with methyl cholate (MeC) and methyl lithocholate (MeLC) in chloroform at 37°C has been studied by vapor pressure osmometry (VPO). This study is part of a larger project to investigate the effect of number and position of hydroxyl-bearing steroids. Using theories developed by Adams and by Steiner, the model and appropriate parameters for the nonideal mixed associations were elucidated. For the MeLC/cholesterol system, no mixed association was observed. For the MeC/cholesterol system, both methods of analysis indicate that a nonideal AB complex formation occurs. The best parameters to explain the experimental data are $k_{AB} = 0.04$ l/g; B_{AB} (the nonideal term) $= 1.5 \times 10^{-5}$ l mol g^{-2}.

1. Introduction

Because of the obvious biological significance, it would be desirable to study interactions of cholesterol with other biochemical constituents in an aqueous environment. However, cholesterol by itself is virtually insoluble in pure water [1], and simulation of an 'in vivo' environment would require a combination of bile salts and lecithins in buffer solution. Since there is no adequate theory for quantitative thermodynamic analysis of such complex, multicomponent solution behavior, a simplified approach to the problem was sought. The alternative chosen was to study the interactions of bile acid esters with cholesterol in pure, organic solvents. Here one is dealing with a two-solute system, and the state of aggregation of each solute in the chosen solvent can be ascertained. In previous publications, we have reported on the self-association of cholesterol [2] and of the methyl esters of lithocholic (MeLC), deoxycholic (MeDC) and cholic (MeC) acids in $CHCl_3$ and CCl_4 [3]. Here we report on the interactions of MeLC and MeC with cholesterol in $CHCl_3$ solutions.

Although mixed associations are quite important in biology and chemistry, studies of mixed associations by thermodynamic methods are rare. This is probably a result of the effort needed to extract the desired information. Some references to earlier studies on mixed associations are given in the papers by Pekar et al. [4] and by Adams et al. [5]. Servillo et al. [6] have reported on the mixed association of apolipoproteins A-II and C-I using sedimentation equilibrium experiments.

The studies reported here were done by vapor pressure osmometry (VPO). We chose VPO because the experimental technique is simpler than ultracentrifugation or light scattering. Ultracentrifugation would be complicated by the compressibility of the solvent, and one would have to determine partial specific volumes and refractive index increments for the reactants, as well as esti-

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Abbreviations: MeC, methyl cholate; MeLC, methyl lithocholate; VPO, vapour pressure osmometry; SEK, sequential equal equilibrium constant.

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mate these quantities for any complexes that might be formed. The solutes in this study are rather small for light scattering, and one would also have to determine refractive index increments for the various solute species present. VPO is a well established technique, and was used quite elegantly with near-infrared spectroscopy by Nagel and Hanlon in their studies of the self-associations of 9-ethyladenine and 1-cyclohexyluracil at 25°C in $CHCl_3$ [7], and also in their study of the mixed association of these compounds in the same environment [8]. These studies and most other previous studies of mixed associations have assumed ideal behavior is present. In this paper we attempt to correct for nonideal behavior using procedures suggested by Adams et al. [5], and similar procedures which have been advocated by Steiner [9,10].

2. Experimental

2.1. Materials

Cholesterol, cholic acid and lithocholic acid were obtained from Sigma Chemical Co. (Sigma grade 99 + %) and were found to be chromatographically homogeneous. Cholesterol was used as received. The methyl esters of cholic acid and lithocholic acid were prepared using Hoffman's method [11], then recrystallized at least twice from methanol and dried under high vacuum at 40°C for several hours. Benzil used to determine the calibration constant for the VPO was purchased from Eastman Kodak Co. and dried under high vacuum at 40°C for approx. 4 h. All solvents were of the highest spectral quality available from Fisher Scientific Co. and/or MCB Manufacturing Chemists, Inc.

2.2. Procedure

VPO experiments were carried out on a Knauer Vapor Pressure Osmometer equipped with the Knauer Universal Temperature Measuring Apparatus and a chart recorder. The operating procedure followed is described in earlier works [2,3,5]. Benzil was used as an external calibration standard. Because the monomer molecular weights of cholesterol and the two methyl esters are known, they were used as internal standards. The results were comparable in all cases. Stock solutions having a definite molar (β_m) or weight proportion (β_g) of the reactants were prepared. Here β_g is defined by

$$\beta_g = c_A^0/c_B^0 \tag{1}$$

where c_A^0 and c_B^0 are the initial concentrations of A and B in g/l. At constant β_g (or β_m) experiments were carried out on the stock solution and a series of dilutions prepared from it. Stock solutions of cholesterol/MeLC in $CHCl_3$ were prepared corresponding to β_g values of 1.02, 1.64 and 1.68. 12–15 dilutions were prepared from each stock solution so that each set of experiments involved varying the total concentration at constant β_g. The cholesterol/MeC in $CHCl_3$ system was studied at values of $\beta_g = 0.458$, 0.915 and 1.83. Because the self-association behavior of the individual components is known [2,3] the data for $\beta_g = 0$ were also available. All measurements were conducted with the measuring cell thermostatically maintained at 37°C.

3. Quantities needed for the analysis

3.1. Interpreting experimental results

The apparent number average molecular weight, M_{na}, is obtained from the VPO experiment using the relation

$$\Delta E = K_{vp}(c/M_{na}) \tag{2}$$

where ΔE is the microvolts imbalance of the Wheatstone bridge circuit on the VPO apparatus, K_{vp} the calibration constant, and c the total solute concentration in g/l. The quantity K_{vp} is usually determined from a calibration experiment with solutions of a nonvolatile, nonassociating solute of known molecular weight; K_{vp} depends on the temperature and the solvent, and it may have a slight dependence on the molecular weight of the solute chosen for the calibration [12]. To avoid this complication one can choose a calibration solute with a molecular weight close to the number average molecular weight of the mixture of A and B (the

two compounds of interest) at infinite dilution (M_n^0). Thus

$$\lim_{\substack{c \to 0 \\ \beta_g = \text{const}}} \Delta E/c = K_{vp}/M_n^0 \tag{3a}$$

or

$$\lim_{\substack{c \to 0 \\ \beta_g = \text{const}}} d\Delta E/dc = K_{vp}/M_n^0. \tag{3b}$$

It should be noted that M_n^0 is also the number average weight of a mixture of A and B under conditions of no association; thus, at constant β_g

$$M_n^0 = \frac{(1+\beta_g)M_A M_B}{\beta_g M_B + M_A} \tag{4a}$$

since

$$c = c_A^0 + c_B^0 = (1+\beta_g)c_B^0 = (1+\beta_g)c_A^0/\beta_g \tag{4b}$$

and

$$\frac{c}{M_n^0} = \frac{c_A^0}{M_A} + \frac{c_B^0}{M_B} \tag{4c}$$

3.2. Evaluation of M_{na}^*

Analysis of a mixed association requires that the self-association behavior of the reactants in the chosen solvent be known. The self-association model, as well as the equilibrium constants and nonideal terms, must be accurately evaluated. Such studies have been conducted on MeLC, MeC and cholesterol in $CHCl_3$ solutions at various temperatures [2,3]. Table 1 shows the values of the equilibrium constants and nonideal terms obtained at 37°C for these systems.

Mixed associations are equilibria of the type

$$nA + mB \rightleftarrows A_nB_m \ (n,m = 1,2,\ldots) \tag{5}$$

$$A + B \rightleftarrows AB$$

$$nA \rightleftarrows A_n$$

and related equilibria. In evaluating associations of this nature it is assumed that the natural logarithms of the activity coefficients are defined as follows

$$\ln y_A = B_{AA}^* M_A c_A^0 + B_{AB}^* M_A c_B^0 \tag{6a}$$

$$\ln y_B = B_{BB}^* M_B c_B^0 + B_{AB}^* M_B c_A^0 \tag{6b}$$

$$\ln y_{A_nB_m} = n \ln y_A + m \ln y_B \tag{6c}$$

for concentrations in g/l. The B_{AA}^* and B_{BB}^* terms are constants whose values depend on temperature and solute-solvent interactions. The assumption in eq. 6c for evaluating mixed associations is analogous to that used in analyzing self-associations, namely

$$\ln y_{A_n} = n \ln y_A. \tag{7}$$

The basis for and validity of these assumptions has been discussed elsewhere [13,14]. Note that $B_{BA}^* = B_{AB}^*$ [5].

The quantity c/M_{na} for mixed associations is defined by

$$c/M_{na} = c/M_n^{eq} + \tfrac{1}{2}\left[B_{AA}(c_A^0)^2 + B_{BB}(c_B^0)^2 \right] + B_{AB}c_A^0 c_B^0. \tag{8}$$

Here

$$B_{AA} = B_{AA}^* + \bar{v}_A/1000M_A \tag{9a}$$

$$B_{BB} = B_{BB}^* + \bar{v}_B/1000M_B \tag{9b}$$

$$B_{AB} = B_{AB}^* + \bar{v}_A/1000M_B + \bar{v}_B/1000M_A \tag{9c}$$

Table 1

Self-association parameters for the individual solutes (in $CHCl_3$ at 37°C)

Compound	Mode of association	Equilibrium constants	Nonideal term (l/g)
Cholesterol	Monomer-dimer	$k_2 = (1.40 \pm 0.05) \times 10^{-2}$ l/g	$BM_1 = (4.67 \pm 0.03) \times 10^{-3}$
	Type-II SEK	$k = (8.60 \pm 0.05) \times 10^{-3}$ l/g	$BM_1 = (6.11 \pm 0.03) \times 10^{-3}$
MeC	Monomer-dimer-trimer	$k_2 = (3.44 \pm 0.10) \times 10^{-2}$ l/g $k_3 = (7.27 \pm 0.07) \times 10^{-3}$ l^2/g^2	$BM_1 = (3.96 \pm 0.16) \times 10^{-4}$
MeLC	Nonideal-nonassociating		$BM_1 = 0.116$

where B_{AA} and B_{BB} are the nonideal terms obtained from analysis of the self-associations of A and B, and $\bar{v}_A$ and $\bar{v}_B$ the partial specific volumes of A and B, respectively. A derivation of eq. 8 is outlined in Appendix A. Since B_{AA} and B_{BB} as well as c_A^0 and c_B^0 are known, eq. 8 can be used to give a new quantity

$$c/M^*_{na} \equiv c/M_{na} - \tfrac{1}{2}\left[B_{AA}(c_A^0)^2 + B_{BB}(c_B^0)^2 \right] = c/M_n^{eq} + B_{AB}c_A^0c_B^0. \quad (10)$$

M_n^{eq} is the true number average molecular weight under conditions of chemical equilibrium. The formulation of c/M_n^{eq} depends on the type of association present. The analytical procedure used to obtain M^*_{na} and evaluate the equilibrium constants and nonideal terms is based on procedures described earlier [4,5].

3.3. Evaluation of X_A^* and X_B^*

In 1968, Steiner [9] published a very elegant method for analyzing ideal mixed associations and then extended his methods to the nonideal case [10]. Although his treatment is based on concentrations in molalities, it can be applied to molar concentration data, provided the compressibility of the solutions is negligible or that the experiments are performed at constant pressure. With aqueous solutions this is no problem, since water is relatively incompressible, but it can be a problem with organic solvents unless the experiments are conducted at constant pressure. This is the case in VPO, since the experiments are done at atmospheric pressure.

Briefly, Steiner [9,10] has shown that the following equations apply:

$$\phi^{**} \equiv \ln Z_A^{**} + \beta_m \ln Z_B^{**} = \int_0^{q^{**}} [(g^* - 1)/q^{**}]\,dq^{**} + \ln\left[\frac{1}{1+\beta_m}\right] + \beta_m \ln\left[\frac{\beta_m}{1+\beta_m}\right] \quad (11)$$

where $\beta_m \equiv q_{B,t}/q_{A,t}$ and $q_{A,t}$ and $q_{B,t}$ are initial molar concentrations of A and B; Z_A^{**} and Z_B^{**} are the apparent equilibrium number fractions of uncomplexed A and B, given by:

$$Z_A^{**} = X_A^{**}q_t/q^{**} = q_A^{**}/q^{**} \quad (12a)$$

$$Z_B^{**} = X_B^{**}q_t/q^{**} = q_B^{**}/q^{**}. \quad (12b)$$

Note that X_A^{**} and X_B^{**} are the apparent stoichiometric number fractions of monomeric A and B and that q_t is the total molar concentration of both species that was initially entered into the solution (c/M_n^0); q^{**} is the apparent equilibrium molarity (i.e., c/M^*_{na}) given by eq. 10; q_A^{**} and q_B^{**} are apparent molar concentrations of uncomplexed A and B; finally, g^* is the apparent osmotic coefficient, or M_n^0/M^*_{na}.

Thus, ϕ^{**} is obtained by carrying out the required integral using the right-hand side of eq. 11, and the same c/M^*_{na} data used in Adams' method (see eq. 10), provided B_{AA} and B_{BB} are known. In order to get Z_A^{**} and Z_B^{**} and thereby X_A^{**} and X_B^{**}, and ultimately q_A^{**} and q_B^{**} (using eqs. 12), we use the following relation:

$$\left(\frac{d\phi^{**}}{d\beta_m}\right)_{q^{**}} = \ln Z_B^{**}. \quad (13)$$

Finally, Steiner has shown that the 'true' or equilibrium molar concentrations of uncomplexed A and B are related to the apparent values by:

$$q_A = q_A^{**} \exp(-B_{AB}M_Ac_B^0) \quad (14a)$$

$$q_B = q_B^{**} \exp(-B_{AB}M_Bc_A^0). \quad (14b)$$

The method for solving for K_{AB} and B_{AB} is discussed in section 4.3.

4. Results

4.1. The mixed association model

If the stoichiometry of the mixed association is not known a priori, then one must formulate models for various possibilities. With the cholesterol/MeC system we assumed that AB and AB_2 complexes might be present; here A ≡ cholesterol and B ≡ methyl cholate. Thus, the first model assumed that

$$2A \rightleftarrows A_2$$
$$5B \rightleftarrows B_2 + B_3$$
$$A + B \rightleftarrows AB$$
$$AB + B \rightleftarrows AB_2 \quad (15)$$

were all occurring simultaneously. For this case the following relations apply:

$$c/M_n^{eq} = c_A/M_A + c_B/M_B + k_{A_2}c_A^2/2M_A + k_{B_2}c_B^2/2M_B + k_{B_3}c_B^3/3M_B + k_{AB}c_Ac_B/M_{AB} + k_{AB_2}c_Ac_B^2/M_{AB_2} \quad (16)$$

$$c/M_n^0 = c_A^0/M_A + c_B^0/M_B = c_A/M_A + c_B/M_B + 2k_{A_2}c_A^2/2M_A + 2k_{AB}c_Ac_B/M_{AB} + 2k_{B_2}c_B^2/2M_B + 3k_{B_3}c_B^3/3M_B + 3k_{AB_2}c_Ac_B^2/M_{AB_2} \quad (17)$$

since

$$c_A^0 = c_A + k_{A_2}c_A^2 + k_{AB}c_Ac_B(M_A/M_{AB}) + k_{AB_2}c_Ac_B^2(M_A/M_{AB_2}) \quad (18)$$

and

$$c_B^0 = c_B + k_{B_2}c_B^2 + k_{B_3}c_B^3 + k_{AB}c_Ac_B(M_B/M_{AB}) + k_{AB_2}c_Ac_B^2(2M_B/M_{AB_2}). \quad (19)$$

The quantity $\Delta(c/M_{na}^*)$ becomes

$$\Delta(c/M_{na}^*) \equiv (c/M_n^0) - (c/M_{na}^*) = -B_{AB}c_A^0c_B^0 + k_{A_2}c_A^2/2M_A + k_{B_2}c_B^2/2M_B + 2k_{B_3}c_B^3/3M_B + k_{AB}c_Ac_B/M_{AB} + 2k_{AB_2}c_Ac_B^2/M_{AB_2}. \quad (20)$$

Since M_n^{eq} depends on both the total concentration of solute and also on β_g ($\beta_g \equiv c_A^0/c_B^0$), a series of experiments at constant β_g values are performed. If this procedure is followed one notes that

$$\lim_{\substack{c\to 0 \\ \beta_g = \text{const}}} \Delta(c/M_{na}^*)/c_A^0c_B^0 = -B_{AB} + k_{A_2}\beta_g/2M_A + k_{B_2}/2\beta_gM_B + k_{AB}/M_{AB} = F(\beta_g) \quad (21)$$

Because k_{A_2} and k_{B_2} are known from experiments on solutions containing only A or B, one can rearrange eq. 21 to give

$$\Lambda = F(\beta_g) - k_{B_2}/2\beta_gM_B - k_{A_2}\beta_g/2M_A = -B_{AB} + k_{AB}/M_{AB}. \quad (22)$$

To evaluate k_{AB}, it is necessary to examine the limiting slopes of plots of $\Delta(c/M_{na}^*)/c_A^0c_B^0$ vs. c at constant β_g. Here one finds that

$$\lim_{\substack{c\to 0 \\ \beta_g = \text{const}}} \left(\frac{\partial}{\partial c}\right)_{\beta_g} [\Delta(c/M_{na}^*)/c_A^0c_B^0] = (\beta_g/1+\beta_g)\{(-k_{A_2}\beta_g/2M_A)[2k_{A_2} + 2k_{AB}M_A/\beta_gM_{AB}] - (k_{B_2}/2M_B\beta_g)[2k_{B_2}/\beta_g + 2k_{AB}M_B/M_{AB}] - (k_{AB}/M_{AB})[(k_{B_2}/\beta_g) + k_{AB}M_A/\beta_gM_{AB} + k_{A_2} + k_{AB}M_B/M_{AB}] + 2k_{AB_2}/\beta_gM_{AB_2} + 2k_{B_3}/\beta_g^2 3M_B\} \equiv L. \quad (23)$$

Eq. 23 involves two unknowns, k_{AB} and k_{AB_2}, and is quadratic in k_{AB} and linear with respect to k_{AB_2}. Thus, if the limiting slope (from eq. 23) is known for two values of β_g, then k_{AB_2} can be eliminated and the resulting quadratic solved to get k_{AB}. This value is then used in eq. 22b to obtain the nonideal term, B_{AB}. Although there are often difficulties in extracting the limiting slopes and intercepts required by eqs. 23 and 21, there are various means of testing these values for internal consistency. For example, Λ, as defined by eqs. 21 and 22 is determined from the intercepts of plots required by eq. 23. These intercepts, when adjusted by the k_{A_2} and k_{B_2} terms given by eq. 22, should give the same value of Λ for every value of β_g. Another method, which allows one to verify the absolute accuracy and the internal consistency of the data in the low-concentration region, is based on eqs. 24 and 25:

$$\lim_{\substack{c\to 0 \\ \beta_g = \text{const}}} \left[\frac{c/M_{na}^* - c_A^0/M_A}{c_B^0}\right] = \frac{1}{M_B} \quad (24)$$

$$\lim_{\substack{c\to 0 \\ \beta_g = \text{const}}} \frac{\partial}{\partial c}\left[\frac{c/M_{na}^* - c_A^0/M_A}{c_B^0}\right] = \frac{\beta_g}{1+\beta_g}\left[\frac{k_{A_2}\beta_g}{2M_A} - \frac{k_{B_2}}{2M_B\beta_g} - \frac{k_{AB}}{M_{AB}} + B_{AB}\right]. \quad (25)$$

Thus, plots of $\left[\frac{c/M_{na}^* - c_A^0/M_A}{c_B^0}\right]_{\beta_g}$ vs. c have a known intercept from eq. 24 and a limiting slope given by eq. 25 which can be rearranged to give $k_{AB}/M_{AB} - B_{AB}$, or Λ in eq. 22. We constructed these plots for the MeC/cholesterol systems (see fig. 1) and found that they were easily fitted by a quadratic least-squares program. The intercepts

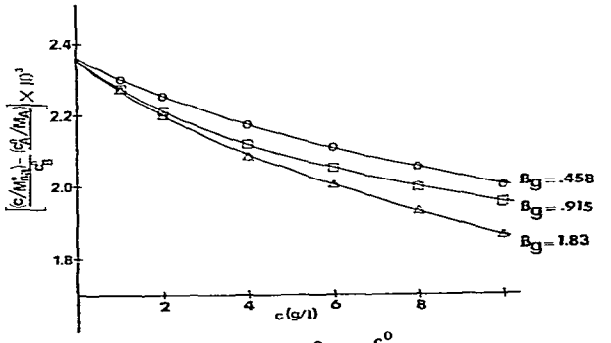

Fig. 1. Plots at constant β of $[\frac{c}{M_{na}^{\circ}} - \frac{c_A^0}{M_A}]/c_B^0$ vs. c (see eqs. 24 and 25) for three blends of cholesterol and MeC in $CHCl_3$ at 37°C. These plots have a common intercept of $1/M_B$ (B = MeC). They are used in the determination of k_{AB} and B_{AB}.

deviated from $1/M_B$ by less than 1% in all cases (see table 2).

Finally, internal consistency of the results can be verified by performing experiments at more than two values of β_g, since only two experiments are required for a solution for k_{AB_2}, k_{AB} and B_{AB} using eqs. 22 and 23. If experiments are conducted at n values of β_g, there are $n!/2!(n-2)!$ unique pairs of experiments which should all give comparable solutions to k_{AB_2}, k_{AB} and B_{AB}. If any of these tests give widely varied results, then either the data are suspect, or the chosen model has failed to describe the system.

4.2. Results using Adams' method

The cholesterol/MeLC system was the first one studied. The results suggest that the net effect of the blend is to form a 'quasi-ideal' solution, with the osmotic coefficient of unity essentially unchanged throughout the concentration range studied (see fig. 2).

Table 2

Results from plots in fig. 1

	Intercept ($\times 10^3$)	% deviation from $1/M_B$ (M_B = 422.59)
0.458	2.349	0.74
0.915	2.358	0.35
1.83	2.354	0.53

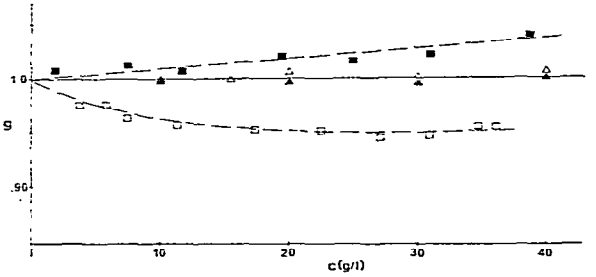

Fig. 2. Plots of the osmotic coefficients (g) vs. c for cholesterol (□), MeLC (■) and two blends of cholesterol and MeLC (β_g = 1.02 (△) and β_g = 1.64 (▲)) in $CHCl_3$ at 37°C. Note that an ideal solution (g = 1) is formed by the two cholesterol/MeLC blends. The plot of g vs. c for MeLC in $CHCl_3$ indicates that there is no self-association, and that the solution is slightly nonideal. The plot of g vs. c for cholesterol in $CHCl_3$ is characteristic of a self-associating solute: for self-associations $g = M_1/M_{na}$.

Fig. 3 shows the g ($= M_n^0/M_{na}$ for mixed associations) vs. c (g/l) data resulting from experiments on the MeC/cholesterol blends at three values of β_g, together with the g vs. c ($g = M_1/M_{na}$ for self-associations) curves for the pure components (all experiments in $CHCl_3$ at 37°C). Because both components self-associate (see table 1) and because the nonideal effects associated with the

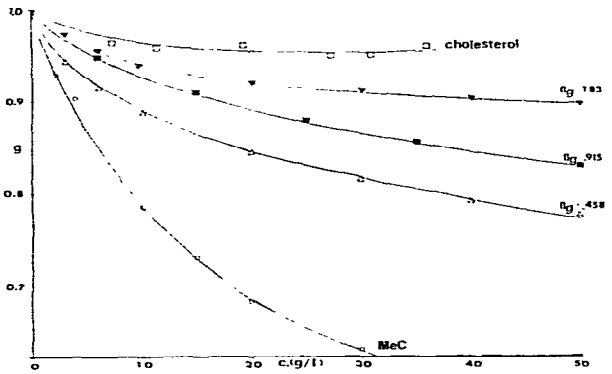

Fig. 3. Plots of g vs. c for cholesterol (□), MeC (○) and three blends of MeC and cholesterol in $CHCl_3$ at 37°C. The curved plots for the three blends indicates that a mixed association is occurring. For mixed associations the osmotic coefficient (g) becomes $g = M_n^0/M_{n\ app}$, where M_n^0 is the number average molecular weight a particular blend of the reactants (cholesterol and MeC) would have if no mixed association occurred.

Table 3

k_{AB} and B_{AB} resulting from AB only model

β_g	k_{AB} (l/g)	B_{AB} (l mol g^{-2})
0.458	0.228	6.64×10^{-5}
0.915	0.146	1.03×10^{-4}
1.83	0.155	8.49×10^{-5}

mixed interactions are not known a priori, no simple inferences as to the presence or absence of a mixed-association were possible. Consequently, the approach taken was to assume a model and then test for its validity, as outlined in section 4.1.

Assuming the possible equilibria shown by eqs. 15, plots of c/M_{na} vs. c were smoothed and the smoothed values used to determine the limiting slopes and intercepts indicated by eqs. 21 and 23. Because we had data for three values of β_g, there were three pairs available for solving for k_{AB} using eq. 23. The results were disappointing – widely varied values were obtained for k_{AB} and negative values for k_{AB_2}. It was decided to test for the presence of a mixed interaction involving AB complex only. The equations were identical with the exception that eq. 23 had no k_{AB_2} terms, consequently there should be one solution for k_{AB} at each value of β_g. The results of this analysis are indicated in table 3, and these results suggest that indeed the AB model may be a valid representation of the experimental system. The next step was an attempt to regenerate the experimental g

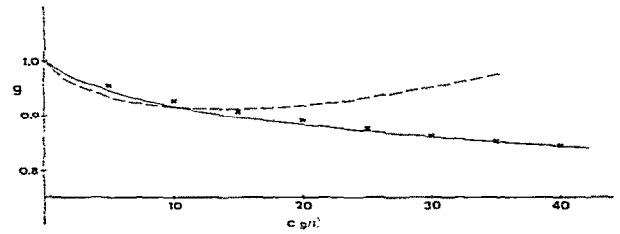

Fig. 4. Experimental and regenerated plots of g vs. c at $\beta_g = 0.915$ for cholesterol/MeC in $CHCl_3$ at 37°C. The experimental points are represented by x. The data regenerated by the Adams method (see table 3) are shown by the dashed line. The data regenerated from the floated values of k_{AB} and B_{AB} (see table 4) are given by the solid curve.

Table 4

Best values of k_{AB} and B_{AB} by variance criterion

β_g	k_{AB} (l/g)	B_{AB} (l mol g^{-2}) ($\times10^5$)	Variance ($i=20$) ($\times10^4$)
0.458	0.033	1.55	6.56
0.915	0.032	1.40	4.88
1.83	0.054	1.54	1.56
Average	0.040 [a]	1.50	

[a] $K_{AB} = 8.08$ l/mol.

vs. c curves using eqs. 8 and 16 with the equilibrium constants and nonideal terms reported in tables 1 and 3.

The results are shown as the dashed-line plot in fig. 4 for the $\beta_g = 0.915$ data and were similar for the other two data sets. Obviously the theoretically derived parameters do not satisfactorily describe the experimental data. Thus, it was decided to vary the parameters independently until the best regenerated results were obtained. This was done using a variance criterion, where the variance is defined by eq. 26:

$$\text{variance} \equiv \frac{1}{n-p}\sum_{i=1}^{n}[y_i - y_i']^2 \tag{26}$$

where n is the number of data points, p the number of parameters, and y_i and y_i' the experimental and regenerated values of g at each data point, respectively. 20 data points were extrapolated from the smooth curves in fig. 3 and a computer program was used to find the set of k_{AB} and B_{AB} values which gave the lowest variance. The results are given in table 4, and the regenerated curve of g vs. c for the $\beta_g = 0.915$ data is represented by the solid line in fig. 4. Obviously, these parameters give an accurate description of the experimentally observed data.

4.3. Results using Steiner's method

Because no mixed association was encountered with the MeLC/cholesterol system, Steiner's method was not applied to these data. For the MeC/cholesterol system, three models were tested using the theory outlined in section 3.3. These

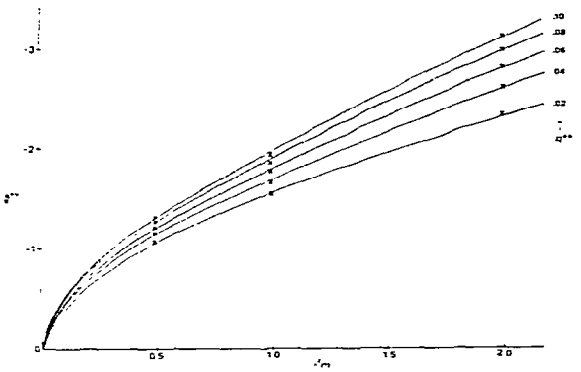

Fig. 5. Plot of ϕ^{**} vs. β_m at constant q^{**}, where $q^{**} = c/M^*_{na}$ is the apparent equilibrium molarity. These plots are used in Steiner's method (see eq. 13) for obtaining Z^{**}_B, the apparent equilibrium number fraction of B. The slope at any point on these curves will give ln Z^{**}_B for the particular values of β_m and q^{**} at that point.

were: (1) mixed association involving AB and AB_2 complexes; (2) mixed association involving AB and A_2B complexes; (3) mixed association involving only AB complex. Application of Steiner's method, though somewhat more mathematically tedious than Adams' approach, offers the advantage of providing a large number of solutions across the concentration range studied. This is best explained by following the procedure step-by-step. As an example, consider the stoichiometry of the $AB + AB_2$ model given by eqs. 15. To apply Steiner's method, we first construct plots of $(g^* - 1)/q^{**}$ vs. q^{**} and integrate from 0 to various values of q^{**} to get ϕ^{**} (see eq. 11). If the integration is carried out for five values of q^{**}, we will have the possibility of five solutions for B_{AB}. This process is repeated at each value of β_m. Next, plots of ϕ^{**} vs. β_m (at constant q^{**}) are constructed for each q^{**} through which the integration was carried out. These plots are shown in fig. 5. The slope of these plots at any β_m gives ln Z^{**}_B by eq. 13. Once Z^{**}_B is known, so is Z^{**}_A from eq. 11, as well as q^{**}_A and q^{**}_B, which are related to the equilibrium mole fractions of uncomplexed A and B by eqs. 14. Finally, we use the mass balance relations and the definition of $q^{**} \equiv c/M^*_{na}$ (given by eq. 10) to arrive at the following relation:

$$q^{**} - q_{A,t} = q_B - k_{A_2} q_A^2 + k_{B_2} q_B^2 + k_{B_3} q_B^3 + \tfrac{1}{2} B_{AB} c_A^0 c_B^0 \qquad (27)$$

where $q_{A,t}$ is the total concentration of A (i.e., the molar equivalent of eq. 18).

If eqs. 12 are used in eq. 27 to substitute $q_A^{**}\exp(-B_{AB}M_A c_B^0)$ for q_A and $q_B^{**}\exp(-B_{AB}M_B c_A^0)$ for q_B, the only unknown in eq. 27 is B_{AB}, which can be solved by successive approximation. Once B_{AB} is known, k_{AB} and k_{AB_2} can be solved from any two of the mass balance equations:

$$q_{A,t} = q_A + 2k_{A_2} q_A^2 + k_{AB} q_A q_B + k_{AB_2} q_A q_B^2 \qquad (28a)$$

$$q_{B,t} = q_B + 2k_{B_2} q_B^2 + 3k_{B_3} q_B^3 + k_{AB} q_A q_B + 2k_{AB_2} q_A q_B^2 \qquad (28b)$$

$$q_{eq} = c/M_n^{eq} = q_A + q_B + k_{A_2} q_A^2 + k_{B_2} q_B^2 + k_{B_3} q_B^3 + k_{AB} q_A q_B + k_{AB_2} q_A q_B^2. \qquad (28c)$$

A similar process results if the $AB + A_2B$ model is

Table 5

Results from Steiner's method

q^{**}	$\beta_m = 0.5$		$\beta_m = 1.0$		$\beta_m = 2.0$	
	k_{AB} (l/mol)	B_{AB} (l mol g^{-2})	k_{AB} (l/mol)	B_{AB} (l mol g^{-2})	k_{AB} (l/mol)	B_{AB} (l mol g^{-2})
0.02	32.6	–	4.6	–	7.6	–
0.04	26.3	–	14.5	–	15.9	–
0.06	26.6	5.9×10^{-5}	19.5	2.3×10^{-5}	18.5	2.5×10^{-6}
0.08	32.4	3.6×10^{-5}	24.6	2.5×10^{-5}	24.4	3.1×10^{-6}
0 10	39.2	4.7×10^{-5}	27.0	1.9×10^{-5}	35.2	6.5×10^{-5}
Average [a]	31.4	2.9×10^{-5}	18.1	1.7×10^{-5}	20.3	4.7×10^{-5}

[a] Average from three β values: $k_{AB} = 23.3$ l/mol (0.115 l/g). $B_{AB} = 3.1\times10^{-5}$ l mol g^{-2}.

assumed. For the 'AB only' model, k_{AB} can be obtained from any one of eqs. 28 (which will not have an AB_2 term) after B_{AB} is found.

Using this process for the AB plus AB_2 model resulted in an average value of $B_{AB} = -6.3 \times 10^{-5}$ and negative solutions for k_{AB_2}. Thus, the model is invalid. Similar results were obtained for the AB plus A_2B model. Finally, the AB only model was tested. The integration described above (see eq. 11) was carried out through q^{**} values of 0.02, 0.04, 0.06, 0.08, and 0.10 (molar) for the three values of β_M. (Note that $\beta_g = 0.458 \Rightarrow \beta_M = 2.0$; $\beta_g = 0.915 \Rightarrow \beta_M = 1.0$; $\beta_g = 1.83 \Rightarrow \beta_M = 0.5$). This provided 15 possible successive approximation solution for B_{AB}, and nine extract solutions were found. Using the average value of B_{AB}, 15 solutions were found for k_{AB}. These results are presented in table 5.

5. Discussion

The results of the experiments on the cholesterol MeLC system are not surprising when one considers the self-association behavior of the individual species. Fig. 2 shows the g vs. c curves of the individual solutes (as dashed lines) with the observed g vs. c data obtained from the mixture at two values of β_g. NMR studies on cholesterol in $CHCl_3$ have indicated that hydrogen bonding of the 3α hydroxyl group is the dominant source of the dimer formation [2]. Since it has been observed that MeLC does not hydrogen bond to itself in $CHCl_3$ [3], it is reasonable to expect it will not associate with cholesterol under these conditions. The experimental results support this prediction – no mixed association is observed.

If the same rationale is applied to the cholesterol/MeC system, one might expect that AB, A_2B and AB_2 complexes are possible, since cholesterol has one site for hydrogen bonding (3α-OH) and MeC has three (3α- , 7α- , 12α-OH). In fact, the results from both methods of analysis (i.e., Adams' [5] and Steiner's [10]) leave little doubt that the AB complex is the only mixed association species present. Although these results may give some insight as to the conformational nature of the various hydroxyl steroids' aggregates in solution, any specific conclusions would be highly speculative. It should be stressed that VPO is a thermodynamic technique with the primary objective in this case being the elucidation of the association model and equilibrium constants – certainly the lack of such quantitative information concerning interactions of this type is quite apparent from searches of the literature.

Table 6

Molar free energies of formation

Process	ΔG_F^0 (kcal/mol)
Dimerization of cholesterol	0.61
Dimerization of MeC	1.22
Trimerization of MeC	3.74
AB complexation of cholesterol and MeC	1.29

Perhaps a more accurate assessment of the relative magnitudes of the various association reactions in the cholesterol/MeC system can be obtained by comparing the free energies of formation, ΔG_F^0, based on the equilibrium constants in tables 1 and 4. These values are given in table 6, and it is obvious that the trimerization of MeC is the dominant association interaction in the system, which considerably complicates the task of elucidating the mixed association model and parameters. Thus, it is encouraging that, although there is considerable scatter in the values of the parameters obtained, both methods lead to the conclusion that the AB only model is the only valid representation of the data obtained. In this case, it was necessary to adjust the theoretically derived values to obtain a satisfactory fit of the observed data. In less complex systems (e.g., no self-association or a simple monomer-n-mer association of only one of the two solutes), either method should give reasonably accurate results directly from the theory. In all cases, it is advisable to verify the accuracy of the results by attempting to regenerate the original data curves.

In previous studies on the self-association of cholesterol [2], we have shown that two models, a monomer-dimer and a Type II SEK indefinite self-association, describe the weak, observed self-association. In this paper we chose to consider

only the monomer-dimer self-association of cholesterol in the analysis of the mixed association between cholesterol and MeC. The reasons for doing this were as follows: (1) The analysis of a mixed association seemed somewhat simpler if only the monomer-dimer model were used. (2) Since a mixed association between cholesterol and MeC occurs, there are two competing equilibria involving the cholesterol; thus, there is relatively less cholesterol available to undergo self-association. For dilute solutions and weak self-associations, it becomes very difficult to distinguish between the two models for the cholesterol self-association. In a future publication we will reexamine this mixed association by including the Type II SEK indefinite self-association of cholesterol. Our purpose here was to study a mixed association under nonideal conditions.

The studies reported here represent one of the first attempts to analyze nonideal mixed associations experimentally. While both Adams' [4,5] and Steiner's [10] methods indicate that only an AB complex is present, the values of the equilibrium constant (k_{AB}) and nonideal term (B_{AB}) by these methods would only describe the lower concentrations data as indicated in fig. 4. To analyze the whole range of the data it was necessary to float the constants with the result shown in fig. 4. The problems we have encountered may be due to the weak association, to our model for nonideal behavior, or to both effects. According to Ogston and Winzor [13], in their study of self-associations, one is trying to find the best average value of the nonideal term (BM_1). Similar conclusions were arrived at by Nichol and Winzor [14] in their theoretical study of the assumption about the activity coefficients being restrained so that $y_{A_nB_m}/y_A^n y_B^n = 1$:. (1) If charge effects dominated the nonideal terms, then this assumption would be obeyed because of conservation of charge. (2) For uncharged spherical molecules using a simulated $A + B \rightleftarrows C$ association, they found that the activity coefficient ratio $y_{AB}/y_A y_B$ differed from 1 by 3.8%. The virtue of using Adams' [5] or Steiner's [10] approach is that the nonideal terms are evaluated from the experimental data without recourse to statistical theories [13–15].

It is possible that a combination of thermodynamic and spectroscopic techniques, such as infrared spectroscopy, may help in deciding on the number of complexes to look for [7,8]. On the other hand, spectroscopic techniques may not be able to detect nonideal effects, which can be detected by appropriate thermodynamic techniques such as VPO, membrane osmometry, sedimentation equilibrium or elastic light scattering [1,2,16]. While there are some parallels between spectroscopic techniques and the thermodynamic techniques mentioned above, it must be remembered that the spectroscopic techniques detect local interactions that can give information about the points of contact, type of bonding, and conformation, but these local interactions may not reflect the behavior of the molecule as a whole, as we have observed in comparing VPO and NMR studies [1,2,16]. Thus, it would seem that VPO and related thermodynamic studies are better suited for testing for the type of association present and for evaluating the values of the equilibrium constant(s) and the nonideal terms. At present, aside from Steiner's [10] elegant theory for evaluating $\ln x_A + \beta \ln x_B$ for mixed associations, and for a special case such as an association of the type

$$A + B \rightleftarrows AB$$
$$AB + AB \rightleftarrows A_2B_2$$
$$AB + A_2B_2 \rightleftarrows A_3B_3$$

etc., there is no interrelation between the number and weight average molecular weight or their apparent values as there is with self-associations. Perhaps these studies will stimulate the discovery of new relations or better ways for studying mixed associations.

Appendix A

To obtain eq. 8 one starts with eq. 186 of ref. 5; thus

$$d/(c/M_{na}) = d(c/M_n^{eq}) + \left(\tfrac{1}{2}\right)\sum_i \sum_j B_{ij}^* d(c_i^0 c_j^0) + \sum_i \sum_j \frac{\bar{v}_i c_i}{1000} d(c_j/M_j). \quad \text{(A1)}$$

Since we do not necessarily know the partial

specific volumes of the associating solutes, and since the last term on the right-hand side of eq. A1 is small, we assume

$$\sum_i \sum_j \frac{\bar{v}_i c_i}{1000} \mathrm{d}(c_j/M_j) \equiv \sum_i \sum_j \frac{\bar{v}_i c_i^0}{1000} \mathrm{d}(c_j^0/M_j). \tag{A2}$$

Here c_i^0 (or c_j^0) is the initial concentration of reactant i (or j) and c_i (or c_j) the equilibrium concentration of the associating species. Substitution of eq. A2 into eq. A1 and integration from $c = 0$ to $c = c$ gives

$$c/M_{\mathrm{na}} = c/M_{\mathrm{n}}^{\mathrm{eq}} + \left(\tfrac{1}{2}\right)\left[B_{\mathrm{AA}}^{*}\left(c_{\mathrm{A}}^0\right)^2 + B_{\mathrm{BB}}^{*}\left(c_{\mathrm{B}}^0\right)^2\right] + B_{\mathrm{AB}}^{*} c_{\mathrm{A}}^0 c_{\mathrm{B}}^0 + \left(\tfrac{1}{2}\right)\left[\frac{\bar{v}_{\mathrm{A}}\left(c_{\mathrm{A}}^0\right)^2}{1000 M_{\mathrm{A}}} + \frac{\bar{v}_{\mathrm{B}}\left(c_{\mathrm{B}}^0\right)^2}{1000 M_{\mathrm{B}}}\right] + \left[\frac{\bar{v}_{\mathrm{A}}}{1000 M_{\mathrm{B}}} + \frac{\bar{v}_{\mathrm{B}}}{1000 M_{\mathrm{A}}}\right]\frac{c_{\mathrm{A}}^0 c_{\mathrm{B}}^0}{2}. \tag{A3}$$

Rearrangement of this equation leads to

$$c/M_{\mathrm{na}} = c/M_{\mathrm{n}}^{\mathrm{eq}} + \left(\tfrac{1}{2}\right)\left[B_{\mathrm{AA}}\left(c_{\mathrm{A}}^0\right)^2 + B_{\mathrm{BB}}\left(c_{\mathrm{B}}^0\right)^2\right] + B_{\mathrm{AB}} c_{\mathrm{A}}^0 c_{\mathrm{B}}^0 \tag{A4}$$

where B_{AA}, B_{BB} and B_{AB} are defined by eqs. 9a–9c. The quantities B_{AA} and B_{BB} are the non-ideal terms for A and B that one obtains from experiments in solutions containing only A or B.

Appendix B

B.1. Some useful relations used in deriving eqs. 21–25

B.1.1. Relation of c and β_g

The total solute concentration can be written as follows:

$$c = c_{\mathrm{A}}^0 + c_{\mathrm{B}}^0. \tag{B1}$$

Let

$$\beta_g = c_{\mathrm{A}}^0/c_{\mathrm{B}}^0 \tag{B2}$$

thus

$$c = c_{\mathrm{B}}^0(1+\beta_g) \tag{B3}$$

and

$$\left(\partial c/\partial c_{\mathrm{B}}^0\right)_{\beta_g} = 1+\beta_g. \tag{B4}$$

Similarly

$$c = c_{\mathrm{A}}^0(1+\beta_g)/\beta_g \tag{B5}$$

$$\left(\partial c/\partial c_{\mathrm{A}}^0\right)_{\beta_g} = \frac{1+\beta_g}{\beta_g}. \tag{B6}$$

B.1.2. Evaluation Of limiting ratios

Using $\mathrm{A} + \mathrm{B} \rightleftarrows \mathrm{AB}$ as an example here and in the section that follows, the total concentration of the reactants can be expressed as

$$c_{\mathrm{A}}^0 = c_{\mathrm{A}} + k c_{\mathrm{A}} c_{\mathrm{B}}\left(M_{\mathrm{A}}/M_{\mathrm{AB}}\right) \tag{B7}$$

$$c_{\mathrm{B}}^0 = c_{\mathrm{B}} + k c_{\mathrm{A}} c_{\mathrm{B}}\left(M_{\mathrm{B}}/M_{\mathrm{AB}}\right). \tag{B8}$$

Thus, one notes

$$\lim_{\substack{c\to 0 \\ \beta_g = \mathrm{const}}} \frac{c_{\mathrm{A}}^0}{c_{\mathrm{A}}} = \lim_{c\to 0}\left(1 + k_{\mathrm{AB}}\frac{c_{\mathrm{B}} M_{\mathrm{A}}}{M_{\mathrm{AB}}}\right) = 1 \tag{B9}$$

$$\lim_{\substack{c\to 0 \\ \beta_g = \mathrm{const}}} \frac{c_{\mathrm{B}}^0}{c_{\mathrm{B}}} = \lim_{c\to 0}\left(1 + k_{\mathrm{AB}}\frac{c_{\mathrm{A}} M_{\mathrm{B}}}{M_{\mathrm{AB}}}\right) = 1. \tag{B10}$$

Since $\beta_g = c_{\mathrm{A}}^0/c_{\mathrm{B}}^0$ one notes

$$c_{\mathrm{B}}^0 = c_{\mathrm{A}}^0/\beta_g = c_{\mathrm{B}}(1 + k c_{\mathrm{A}} M_{\mathrm{B}}/M_{\mathrm{AB}}) \tag{B11}$$

and

$$\lim_{\substack{c\to 0 \\ \beta_g = \mathrm{const}}} \frac{c_{\mathrm{B}}^0}{c_{\mathrm{B}}} = 1/\beta_g \qquad \lim_{\substack{c\to 0 \\ \beta_g = \mathrm{const}}} \frac{c_{\mathrm{A}}^0}{c_{\mathrm{B}}} = 1 \tag{B12}$$

hence

$$\lim_{\substack{c\to 0 \\ \beta_g = \mathrm{const}}} c_{\mathrm{A}}^0/c_{\mathrm{B}} = \beta_g \tag{B13}$$

and

$$\lim_{\substack{c\to 0 \\ \beta_g = \mathrm{const}}} c_{\mathrm{B}}^0/c_{\mathrm{A}} = 1/\beta_g. \tag{B14}$$

These relations can be extended to associations more complicated than $\mathrm{A} + \mathrm{B} \rightleftarrows \mathrm{AB}$.

B.1.3. Limiting derivatives

The total concentration of the reactants can be written as

$$c_{\mathrm{A}}^0 = c_{\mathrm{A}}\left[1 + k c_{\mathrm{B}}\left(M_{\mathrm{A}}/M_{\mathrm{AB}}\right)\right] = \beta_g c_{\mathrm{B}}^0 \tag{B15}$$

and

$$c_B^0 = c_B[1 + kc_A(M_B/M_{AB})] = c_A^0/\beta_g. \quad \text{(B11)}$$

Now note for example that

$$c_A = \beta_g c_B^0/[1 + kc_B(M_A/M_{AB})]. \quad \text{(B16)}$$

Thus

$$c_B^0 = c_B\left[1 + \frac{k(M_B/M_{AB})\beta_g c_B^0}{(1 + kc_B(M_A/M_{AB})}\right] = f(c_B, \beta_g) \quad \text{(B17)}$$

Similarly,

$$\lim_{\substack{c \to 0 \\ \beta_g = \text{const}}} \frac{\partial c_A^0}{\partial c_B} = 1. \quad \text{(B18)}$$

Since $c_A^0 = \beta_g c_B^0$, it follows that

$$\lim_{\substack{c \to 0 \\ \beta_g = \text{const}}} \frac{\partial c_A^0}{\partial c_A} = \lim_{\substack{c \to 0 \\ \beta_g = \text{const}}} \beta_g \frac{\partial c_B^0}{\partial c_A} = 1 \quad \text{(B19)}$$

and

$$\lim_{\substack{c \to 0 \\ \beta_g = \text{const}}} \frac{\partial c_B^0}{\partial c_A} = 1/\beta_g. \quad \text{(B20)}$$

Also

$$\lim_{\substack{c \to 0 \\ \beta_g = \text{const}}} \frac{\partial c_A^0}{\partial c_B} = \beta_g. \quad \text{(B21)}$$

These arguments can be extended to associations more complicated than $A + B \rightleftarrows AB$.

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