Exponential Analysis of Concentration or Concentration Difference Data for Discrete Molecular Weight Distributions in Sedimentation Equilibrium*

Rudy H. Haschemeyer and William F. Bowers†

ABSTRACT: The nonlinear exponential concentration distribution expression for ideal, limited, discrete self-associating and noninteracting systems in sedimentation equilibrium is successfully solved directly for one or more of the molecular weights as well as for the concentrations of all species assumed present. Parallel equations are considered for both true concentration and concentration difference data, and the relative merits of these solutions for a variety of systems are demonstrated with the aid of realistic simulated data. A method is shown for obtaining equilibrium constants of self-associating systems which contain some macromolecules not participating in the chemical equilibrium.

Current methods for analysis of multicomponent systems in sedimentation equilibrium usually involve evaluation of a variety of molecular weight averages. For noninteracting systems these may be used directly to numerically describe limited distributions and may be extrapolated in various ways to obtain derived quantities such as the molecular weight of the smallest component (Yphantis, 1964). Self-associating systems have most often been analyzed by application and extension of equations developed by Steiner (1954) for light scattering. These equations were first applied to ultracentrifuge studies in the approach to equilibirum method by Rao and Kegeles (1958) and in sedimentation equilibrium by Squire and Li (1961). Since then the theory has been extended and applied to a wide variety of systems (*e.g.*, Adams and Fujita, 1963; Adams and Williams, 1964; Nichol *et al.*, 1964; Adams, 1964, 1965, 1967a,b; Adams and Filmer, 1966; Jeffrey and Coates, 1966; Van Holde and Rossetti, 1967; Chun and Fried, 1967; Chun *et al.*, 1968; Adams and Lewis, 1968; Albright and Williams, 1968; Jeffrey, 1968; Derechin, 1968, 1969a,b; Roark and Yphantis, 1968; Teller *et al.*, 1968; Van Holde *et al.*, 1968; Swann and Hammes, 1969; Hoagland and Teller, 1969).

An alternative direct description of discrete molecular weight distributions in sedimentation equilibrium is obtained when the concentration as a function of radial position is expressed as a sum of exponential terms (Svedberg and Pederson, 1940) containing, in the most general case, unknowns to be determined in both the amplitude and exponent of each term. These solutions correspond to the concentration at a reference position and to a quantity proportional to the component molecular weight, respectively. If the exponents are known, as might often be the case for self-associating systems, the equations become linear and have straightforward algebraic solutions (Svedberg and Nichols, 1926; Adams and Williams, 1964).

Computer solution of the overdetermined simultaneous linear equations was first reported by Reinhardt and Squire (1965) in their study of ovine interstitial cell stimulating hormone. Their successful analysis of the linear equations using nonlinear programming was shown to be in good agreement with the results obtained on the data using the Steiner (1954) molecular weight moments approach. The direct noniterative least-squares solution of the linear equations was applied to sedimentation equilibrium studies on the dimerization of α-chymotrypsin by Teller (1965) and by Teller and coworkers (1968). Again, the results were in excellent agreement with those obtained by the Steiner method. These workers also considered the effects of nonideality and incompetence upon self-associating systems.

Van Holde *et al.* (1968) have shown that the relation presented by Steiner (1954) can itself be manipulated to yield sets of overdetermined linear equations in terms of point molecular weight averages as a function of concentration. They include a nonideality treatment similar to the method we have suggested in this work, solving the nonlinear equations directly for the nonideality constant. The equations developed by these authors were quite successful in describing the self-association of cytidine and purine and will merit consideration as the possible method of choice for analysis of a wide range of other self-associating systems where the monomer molecular weight is known with accuracy.

It has been noted in several of the above references (Reinhardt and Squire, 1965; Teller, 1965; Teller *et al.*, 1968) that unsatisfactory solutions of the linear equations are often characterized by physically unreal negative values of concentration. In an effort to circumvent the problem, Reinhardt and Squire (1965) recommended forcing nonlinear iteration to exclude negative values of concentration. Although they were able to demonstrate a negligible deviation of experimental parameters from those obtained by back-calculation from their solution, the procedure might sometimes be hazardous since we are aware of no simple method of nonlinear programming which permits iterative convergence to an an-

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swer which assures the optimization of some *a priori* selected objective function while simultaneously incorporating constraint conditions.

Linear programming, on the other hand, does allow such linear constraints to be imposed and was very successfully applied by Scholte (1968) to the related problem of fitting sedimentation equilibrium data for broad, continuous molecular weight distributions to a limited sum of exponential terms containing appropriately preset values for the exponent. It seems likely that this procedure permits valid extraction of more unknown parameters than is possible from the more conventional simultaneous solution of linear equations, but satisfactory methods for expressing nonideal behavior in terms of additional linear unknowns are still lacking.

In the most general case of limited discrete mixtures in sedimentation equilibrium, whether interacting or not, a serious difficulty is encountered because of the limited precision with which the molecular weight terms in the exponent can be independently established. It is a major purpose of this work to show how and under what conditions these quantities can be evaluated from concentration distribution data by considering one or more molecular weights as additional unknowns and solving the resultant nonlinear equations directly. Our procedure involves classical methods of numerical analysis which have long been known to be applicable to sedimentation equilibrium in principle. However, only Reinhardt and Squire (1965) have reported attempting the nonlinear solution. Their failure to uniquely define the monomer molecular weight of a self-associating system using the nonlinear equations may have resulted from too modest a centrifugal redistribution and/or from attempting to fit the data to an excessive number of unknown parameters.

A second objective of the work presented here is to show that a simple manipulation of the exponential equations converts them into a form which can utilize concentration difference data directly and which will still enable adequate evaluation of a limited number of unknown concentrations and/or molecular weights. As will be shown, these solutions are less satisfactory than those obtained from data where the concentration itself may be evaluated with precision, but should provide a useful alternative in cases where absolute concentration is excessively difficult to obtain.

Computer-simulated realistic data are used to compare the relative desirability of employing the linear and nonlinear equations expressed in both concentration and concentration difference form for a variety of ideal paucidisperse systems. Direct algebraic solution of the equations to evaluate a frequently encountered type of incompetence for self-associating systems is also presented.

Fitting an experimentally derived curve to a sum of exponentials with unknown amplitudes and exponents (some of which may be constrained to predetermined values) has also been the subject of considerable attention in studies of electrical circuitry, compartmental analysis, radioactive decay, etc. As a result, several applicable numerical algorithms are to be found in standard textbooks of numerical analysis (*e.g.*, Lanczos, 1956; Hildebrand, 1956; Faddeeva, 1959; Pennington, 1965; McCalla, 1967). *A priori* problems that may be encountered in these solutions are discussed in detail by Lanczos (1956) and Hildebrand (1956). They arise mainly from the fact that the vector coordinate system implied is highly skew angular and that the simultaneous equations represent a nearly singular system, regardless of the particular formulation attempted for solution. It follows that the ability to successfully perform the separation of exponentials decreases rapidly with increasing experimental error. The solution becomes more difficult as the number of unknown parameters increases, and even for a limited number of parameters a meaningful solution may be unattainable as the values of the exponents either approach one another, or are too far apart, or as the amplitude of one component becomes small with respect to the other. In this study we have quantitatively determined these limits by choosing several typical systems and demonstrating the stability toward experimental error of representative numerical solutions.

The equations that are solved here have been extensively discussed in the literature cited and are summarized below. We also rewrite them in a form compatible with concentration difference, ΔC, data, which may be directly obtained from the Rayleigh interference pattern at sedimentation equilibrium.

Sedimentation–diffusion equilibrium of an ideal solute, i, is described by the equation

$$\frac{\mathrm{d} \ln C_i}{\mathrm{d}X^2} = \frac{M_i(1 - \bar{V}_i\rho)\omega^2}{2RT} \equiv H_i \tag{1}$$

(Svedberg and Pederson, 1940; Schachman, 1958). In the equation, C_i is the concentration at the radial position X and $\bar{V}_i$ is the partial specific volume of the solute i of molecular weight M_i. R and T are the gas constant and absolute temperature, respectively, and ρ is the solution density, which is assumed to be independent of the centrifugal field. The equation provides the definition of H_i. The total concentration at each radial position, $C(X)$, is the sum of the individual i solute components, all concentrations being expressed in units measured by the optical system employed. Equation 1 may be integrated between the radial position corresponding to the meniscus, X_m, and X to give the concentration distribution

$$C(X) = \sum_i C_{im} e^{H_i(X^2 - X_m^2)} \tag{2a}$$

or alternatively

$$C(X) = \sum_i C_{im} e^{iH(X^2 - X_m^2)} \tag{2b}$$

if the ith species has a value of H which is i times that of the first solute, as is the case for self-associating systems, assuming $\bar{V}$ to be unchanged by polymerization. For ideal self-associating systems the relation between total concentration and that of each component may be written in terms of the monomer concentration C_i, and the equilibrium constant for the ith association as

$$C = \sum_i K_i C_1^i \tag{3}$$

where K_1 is always unity. The sedimentation equilibrium distribution for the purely self-associating system in chemical equilibrium is given by

$$C(X) = \sum_i K_i C_{1m}^i e^{iH(X^2 - X_m^2)} \tag{4}$$

More complex mechanisms involving multiple forms of monomer with different association constants, association between nonidentical units, or inclusion of terms for incompetent species (*i.e.*, solute components of essentially static proportions and unable to enter into the equilibrium due to partial denaturation, etc.) are readily written in similar fashion. In the latter case, which is of particular interest in our laboratory, we may consider the concentration of the ith species to be the sum of competent, CC, and incompetent, CI, protein species at all radial positions. C_{im} in eq 2b is then given by

$$C_{im} = CI_{im} + CC_{im} = CI_{im} + CC_{1m}{}^{i}K_i \tag{5}$$

and the concentration distribution is obtained by substituting eq 5 into eq 2b

$$C(X) = \sum_i (CI_{im} + CC_{1m}{}^{i}K_i)e^{iH(X^2 - X_m{}^2)} \tag{5a}$$

A statement of conservation of mass requires that

$$\int_{X_m}^{X_b} C(X)\mathrm{d}X^2$$

be unchanged as the result of centrifugal distribution. Since the integral has the value $C_0(X_b{}^2 - X_m{}^2)$ before redistribution has occurred, the initial concentration, C_0, is

$$C_0 = \frac{1}{X_b{}^2 - X_m{}^2}\int_{X_m}^{X_b} C(X)\mathrm{d}X^2 \tag{6}$$

where the right-hand side is readily evaluated by substituting the desired equation for $C(X)$. As emphasized by Adams (1964), it is necessary to differentiate between the non- and self-associating expression for conservation of mass in the following way. For nonassociating systems, the analogy of eq 6 can be written separately for each of the i components, *i.e.*

$$C_{i0} = \frac{1}{X_b{}^2 - X_m{}^2}\int_{X_m}^{X_b} C_i(X)\mathrm{d}X^2 = C_{im}\left[\frac{e^{H_i(X_b{}^2 - X_m{}^2)} - 1}{(X_b{}^2 - X_m{}^2)H_i}\right] \tag{6a}$$

while for associating systems we have instead

$$C_0 = \sum_i C_{1m}{}^{i}K_i\left[\frac{e^{iH(X_b{}^2 - X_m{}^2)} - 1}{(X_b{}^2 - X_m{}^2)iH}\right] = \sum_i C_{1m}{}^{i}K_iE_i \tag{6b}$$

where the relation between the ith-mer and C_{i0} can only be found by solution of the nth-order equation and the term in brackets serves to define a convenient quantity, E_i. A numerical solution for C_{im} in eq 6b always exists if the K_i are known, but no closed form solution is otherwise available (in terms of unknown K_i) for the general case with n greater than 4.

An expression for the concentration of the ith purely self-associating component at sedimentation equilibrium under one set of experimental conditions may readily be related to that at another if an overlap in concentration is present. A reference radial position may then be chosen in each of two experiments such that $C(X_r) = C(X_r')$, with the numerical value of X_r' determined analytically. Since the concentration of the ith component of a self-associating system is a unique function of concentration, the desired relation can be expressed as

$$C_{1m}{}^{i}K_i = C_{1m}{}'^{i}K_i\frac{e^{iH'(X_r'^2 - X_m'^2)}}{e^{iH(X_r{}^2 - X_m{}^2)}} \tag{7}$$

where the primes denote the known value of the designated parameters in the alternate experiment. In this fashion it is possible to incorporate any number of experiments into a single set of equations approximating a least-square solution without increasing the number of unknown parameters.

The distribution obtained from runs at two different values of speed and C_0 have been combined to allow preliminary numerical treatment of the specific case of incompetence in which at least one species may be assumed to be totally competent. Since both the equilibrium constants and the fraction of C_0 representing each incompetent species are the same in both runs, one may directly solve algebraically for incompetent monomer at the meniscus in terms of the totally competent species i

$$CI_{1m} = \frac{C_{1m}'\left(\frac{C_{im}}{C_{im}'}\right)^{1/i} - C_{1m}}{\mathrm{f}\left(\frac{C_{im}}{C_{im}'}\right)^{1/i}\frac{E_1}{E_1'} - 1} \tag{8}$$

where f is defined by $C_0' = \mathrm{f}C_0$. Once CI_{1m} is known we may obtain CI_{1m}' from eq 6a and from these quantities readily find CC_1 and CC_1'. All of the K_i (and subsequently the CI_{i0} from eq 5 and 6a) may then be obtained from

$$K_i = \frac{\mathrm{f}C_{im}\frac{E_i}{E_i'} - C_{im}'}{\mathrm{f}CC_{1m}{}^{i}\frac{E_i}{E_i'} - CC_{1m}{}'^{i}} \tag{9}$$

All of the preceding discussion is equally applicable to concentration difference data. Let $Y(X)$ be defined by

$$Y(X) = C(X) - C_m \tag{10}$$

where $Y(X)$ may be obtained by subtracting the extrapolated microcomparator value at the meniscus from that obtained at other X positions.[1] Equations 2, 4, and 5 are readily converted into ΔC form by subtracting C_m from both sides and rewriting C_m on the right-hand side as the sum of all components at the meniscus. Thus eq 2a may be written in ΔC form as

$$Y(X) = \sum_i C_{im}[e^{H_i(X^2 - X_m{}^2)} - 1] \tag{11}$$

The ΔC form of the other equations is similarly obtained by replacing $C(X)$ by $Y(X)$ and adding "-1" to the coefficients of C_{im}.

[1] This form is used to simplify the equations. In practice it may be more convenient, for example, to reformulate the equations in terms of the radial position corresponding to the first real data point.

TABLE I: Physical Parameters Used for Generation of Stimulated Sedimentation Equilibrium Data.

Parameter	Value
$\bar{V}$	0.758 cc/g
ρ	1.0 g/cc
T	293°K
X_m	5.905 cm
X_b	6.205 cm
C_0	1.6 mm
M_1	60,000
$(dC/dX)_{b,cell}$	40.0 fringes/mm
Revolutions per minute	See text
Simulated error	
Standard deviation	$\pm(0.003 + 1.25 \times 10^{-4} \times (dC/dX)_{cell})$
IX[a]	91,437

[a] Arbitrary odd integer argument for random number generating subroutine "Random" written by Dr. Peter Lewis, T. J. Watson Lab, IBM Corp., Yorktown Heights, N. Y. IX determines the particular set of random numbers used for experimental error simulation. See text for details.

Numerical Methods

Synthetic $C(X)$ *vs.* X^2 data were computed with the aid of the equations presented in the previous section. Input parameters for nonassociating species included C_0 and the fraction of each species present before redistribution; for self-associating systems, total C_0 and the K_i were entered. Certain parameters were maintained constant (Table I) to permit less confused comparisons. The value of ω^2 was always chosen as the highest speed setting on the Spinco Model E ultracentrifuge equipped with photoelectronic speed control consistent with a fringe density at $X_b((dC/dX)_{b,cell})$ of 40 fringes/mm in the cell or less, in accord with our experience for the resolution limit of typical Rayleigh equilibrium patterns. The double precision (16 significant figures) values of $C(X)$ are termed "synthetic data" and are useful *per se* in checking for programming errors and in determining the degree of rounding error at various stages in solution. The units of measurement referred to in the Results section are millimeters and microns. Both refer to fringe displacement on a hypothetical Rayleigh interference plate with a fringe separation of 290 μ.

Actual measurements of Rayleigh interference patterns are limited in precision. Repeated measurements of fringe patterns in our laboratory suggest that a reasonable approximation to actual measurement errors is given by the sum of two factors, one of which is constant throughout the cell with a standard deviation ± 3 μ (± 2 μ in both the base line and equilibrium patterns), and the other of which depends on the curvature of the fringes and is essentially proportional to $(dC/dX)_{cell}$, so that the total standard deviation increases to $\sim\pm 8$ μ at a fringe density of 40 fringes/mm in the cell ($\sim$20 on the plate). The standard deviation was determined from the mean of three white fringes read multiple times at each X position. For high-speed meniscus depletion runs this error analysis is similar to that of Roark and Yphantis (1968). We consider a standard deviation of $\pm(0.003 + 1.25 \times 10^{-4} \times (dC/dX)_{cell})$ mm on the plate to be realistic at all concentrations. "Simulated data" are defined as synthetic data which has superimposed upon it a Gaussian distribution of random error, the standard deviation of which is determined at each point from the above expression. Estimated errors in the absolute determination of C for low- and moderate-speed equilibriums runs are more subjective, and depend in part on both the system being studied and the particular experimental approach used. Unless stated otherwise, this error is not incorporated into the simulated data.

Simultaneous overdetermined linear equations were solved by standard matrix methods (*e.g.*, Lanczos, 1956; Faddeeva, 1959; Pennington, 1965). Nonlinear equations were solved in a least-squares extension of Newton's method by expanding the appropriate equations in one-term Taylor's series containing estimates of parameters and solving the now-linear equations for the error in the estimates. The improved estimates were reentered and the iteration repeated until all errors were negligible (McCalla, 1967; Pennington, 1965). This method for determination of nonlinear parameters will be referred to a LTDC (linear Taylor differential correction) and the determination of linear coefficients will be abbreviated LC.

There exist a number of related numerical methods which do not require estimates for the solution of the nonlinear sum of exponentials given data at equal increments of X^2 (Lanczos, 1956; Hildebrand, 1956). Specifically, we have tested Prony's algorithm (also discussed by Teller (1965) in relation to sedimentation equilibrium experiments) for possible application to paucidisperse systems in sedimentation equilibrium and found it excessively unstable to experimental error, requiring data accurate to 0.1 μ for even simple mixtures of two components differing in molecular weight by a factor of 2. Since the method is of little practical value without stabilizing modifications (*e.g.*, noise filtration of the data with Fourier synthesis (Lanczos, 1956)) no results are given.

Results

Direct solution of eq 2a by LTDC for mixtures where all concentrations and molecular weights are unknown is illustrated for two solute components in Tables II and III. The quality of results for conditions other than those presented can be reasonably inferred from the tables. Any generalized statement concerning the quality of the answer is necessarily subjective and would, in general, be influenced by the nature of the problem and/or by alternate methods available for evaluating the distribution. Nonetheless, such statements may be useful throughout this section, since we have acquired considerably more data than could reasonably be presented in tabular form. With the above reservations, we conclude that solutions obtained for C data contain potentially useful information in all cases given in the tables but that ΔC solutions yield "poor" results for the more challenging cases (*e.g.*, small contamination with a lower molecular weight species or for $M_2 < 2M_1$). The extension of LTDC to solve for three species with six unknown parameters is unsatisfactory. Intermediate cases with some of the concentrations and/or molecular weights known are soluble. We estimate that a total of five unknown parameters can usually be successfully extracted from the data of a single run provided that the molecular weights are not too close together and the concentrations are not too small.

TABLE II: Exponential Analysis of Paucidispersity. Resolving Power of LTDC for Two Components of Constant M.

		True Value		Calculated Values			
				C Data		ΔC Data	
C_{10}/C_0	i	M	C_0	M	C_0	M	C_0
0.05	1	60,000	0.08	70,910	0.14		
	2	120,000	1.52	121,630	1.46	*a*	
0.1	1	60,000	0.16	63,080	0.19	*a*	
	2	120,000	1.44	121,080	1.41		
0.2	1	60,000	0.32	61,390	0.35	83,470	0.65
	2	120,000	1.28	121,110	1.25	128,110	0.92
0.4	1	60,000	0.64	60,160	0.65	67,560	0.77
	2	120,000	0.96	120,090	0.95	125,000	0.82
0.5	1	60,000	0.8	60,070	0.81	65,850	0.92
	2	120,000	0.8	120,950	0.79	125,630	0.67
0.6	1	60,000	0.96	59,860	0.96	63,160	1.03
	2	120,000	0.64	120,660	0.64	124,540	0.56
0.8	1	60,000	1.28	59,770	1.28	61,490	1.32
	2	120,000	0.32	120,280	0.33	125,730	0.27
0.9	1	60,000	1.44	59,730	1.43	60,880	1.46
	2	120,000	0.16	119,290	0.17	127,770	0.13
0.95	1	60,000	1.52	59,740	1.51	60,740	1.53
	2	120,000	0.08	118,220	0.09	134,170	0.06

[a] No meaningful solution.

TABLE III: Exponential Analysis of Paucidispersity. Resolving Power of LTDC for Two Components of Equal Concentration.

		True Value		LTDC Solutions			
				C Data		ΔC Data	
M_1/M_2	i	M	C_0	M	C_0	M	C_0
0.2	1	24,000	0.8	24,020	0.80	27,220	0.76
	2	120,000	0.8	120,480	0.79	121,560	0.78
0.4	1	48,000	0.8	48,080	0.81	53,930	0.85
	2	120,000	0.8	120,800	0.79	123,870	0.72
0.6	1	72,000	0.8	72,020	0.82	79,520	1.04
	2	120,000	0.8	121,110	0.78	129,190	0.55
0.8	1	96,000	0.8	97,110	0.95	106,840	1.55
	2	120,000	0.8	124,270	0.64	190,390	0.03

Limited molecular weight distributions described by multiples of a fundamental H may be evaluated from eq 2b in general, or specifically from eq 4 for self-associating systems in equilibrium. Such systems reduce the number of coefficients in the LTDC solution to a term for the meniscus concentration of each species plus one for the fundamental H. If all the H_i are known, LC solutions are applicable. Simulated data for associating systems are computed from the desired values of K_i and C_0 (usually 1.6 mm) by solving eq 6b for the C_i. Since there always exists a nonassociating molecular weight distribution which would have given the same sedimentation equilibrium pattern, the dual solutions are reported both in terms of K_i (for the pure association) and, for perspective, in terms of an "analogous C_{i0}" of the corresponding ith nonassociating component computed from eq 2b and 6a.

The results obtained with typical monomer–dimer–trimer associating systems are shown in Table IV. When the distribution contains good representation of all species (Table IV-A), the results are excellent except for the ΔC LTDC solution. The "mode of association" is also readily determined for $i \leqslant 3$ as illustrated by the monomer–trimer case solved for all three components (Table IV-B). Reasonably adequate solutions are also obtained when one species predominates (Table IV-C), until some lower limit of concentration is reached as is

TABLE IV: Exponential Analysis of Self-Association. Three-Component Solutions.

			Solutions							
			C Data				Δ*C* Data			
	True Value		LC		LTDC		LC		LTDC	
i	C_0	*K*	C_0	*K*	C_0	*K*	C_0	*K*	C_0	*K*
A					M_1 = 60,390				M_1 = 63,910	
1	0.62	1.0	0.62	1.0	0.63	1.0	0.61	1.0	0.63	1.0
2	0.50	1.0	0.48	0.94	0.49	0.94	0.50	1.02	0.60	1.09
3	0.49	1.0	0.50	0.98	0.48	0.93	0.49	1.05	0.35	0.63
B					M_1 = 60,400				M_1 = 62,840	
1	0.77	1.0	0.78	1.0	0.79	1.0	0.77	1.0	0.71	1.0
2	0.0	10^{-6}	0[a]	0	0	0	0	0	0.18	0.28
3	0.83	1.0	0.84	0.98	0.82	0.94	0.83	1.03	0.68	1.01
C					M_1 = 59,570				M_1 = 49,130	
1	0.034	1.0	0.041	1.0	0.032	1.0	0.026	1.0	−0.21	1.0
2	1.49	10^3	1.47	6.6×10^2	1.46	1.06×10^3	1.49	1.64×10^3	1.13	0.2×10^2
3	0.079	10^3	0.089	6.2×10^2	0.104	1.47×10^3	0.084	2.23×10^3	0.64	-0.4×10^2
D					M_1 = 60,740				M_1 = 63,740	
1	0.1	1.0	0.11	1.0	0.09	1.0	0.10	1.0	−0.09	1.0
2	0.011	1.0	−0.016	−1.1	0.07	7.33	0	−0.6	0.53	0.52
3	1.49	10^3	1.51	7×10^2	1.43	1.14×10^3	1.5	9.7×10^3	1.12	8.7×10^2

[a] All values of absolute magnitude less than 10^{-2} are reported as "0."

TABLE V: Exponential Analysis of Self-Association. Five-Component LC Solutions.

	True Value		Solution			True Value		Solution	
i	C_0	*K*	C_0	*K*	*i*	C_0	*K*	C_0	*K*
A					D				
1	0.609	1.0	0.613	1.0	1	0.74	1.0	0.75	1.0
2	0.414	1.0	0.409	0.97	2	0.0	10^{-6}	−0.03	−0.05
4	0.247	1.0	0.249	0.98	4	0.45	1.0	0.49	1.01
6	0.181	1.0	0.175	0.93	6	0	0	−0.04	−0.09
8	0.149	1.0	0.154	0.98	8	0.41	1.0	0.42	0.89
B					E				
1	0.591	1.0	0.601	1.0	1	0.167	1.0	0.17	1.0
2	0.390	1.0	0.361	0.89	2	0.031	1.0	0.03	0.98
3	0.282	1.0	0.319	1.07	4	1.40	10^3	1.40	9.5×10^2
4	0.219	1.0	0.198	0.84	6	0	0	0	0.15×10^2
8	0.117	1.0	0.121	0.84	8	0	$\leqslant 10^3$[a]	0	0.5×10^3
C					F				
1	0.559	1.0	0.569	1.0	1	0.38	1.0	0.48	1.0
2	0.367	1.0	0.332	0.87	2	0.15	1.0	−0.07	−0.31
3	0.271	1.0	0.334	1.17	4	0.026	1.0	0.30	4.76
4	0.218	1.0	0.153	0.65	6	0	0	−0.23	−11.8
5	0.185	1.0	0.213	1.05	8	1.05	10^3	1.12	0.2×10^3

[a] All values of K_8 assumed gave approximately the same solution.

TABLE VI: Exponential Analysis of Mixed Distribution. Correction for Incompetence.[a]

			C Data Solutions				ΔC Data Solutions	
	True Value		LC		LTDC		LC	
i	CI_0/C_0	K	CI_0/C_0	K	CI_0/C_0	K	CI_0/C_0	K
					M_1 = 60,230[b]			
1	0.1	1.0	0.106	1.0	0.129	1.0	0.135	1.0
2	0.0	1.0	0.0	0.98	0.0	1.11	0.0	1.27
4	0.1	1.0	0.103	1.03	0.143	0.98	0.082	1.79
					M_1 = 60,230			
1	0.1	1.0	0.074	1.0	0.125	1.0	0.064	1.0
2	0.0	1.0	0.0	0.84	0.0	1.09	0.0	0.94
4	0.1	1.0	0.124	0.65	0.142	0.94	0.109	0.75
8	0.0	0.0		0		0		0

[a] Combined data from C_0 = 1.6 and C_0 = 0.8 runs, with dimer assumed to be totally competent, revolutions per minute = 12,000 and 15,000, respectively. [b] M_t reported is for C_0 = 1.6 run.

TABLE VII: Exponential Analysis of Self-Association. Effect on All Solutions of Random Experimental Error.

				Normal Error						Double Error[b]					
				C Data				ΔC Data		*C* Data				ΔC Data	
		True Value		LC		LTDC		LC		LC		LTDC		LC	
IX[a]	i	C_0	K	C_0	K	C_0	K	C_0	K	C_0	K	C_0	K	C_0	K
						M_1 = 60,300						M_1 = 60,600			
91,437	1	0.637	1.0	0.64	1.0	0.65	1.0	0.61	1.0	0.65	1.0	0.66	1.0	0.59	1.0
	2	0.501	1.0	0.49	0.97	0.49	0.95	0.52	1.13	0.49	0.94	0.48	0.90	0.54	1.27
	4	0.462	1.0	0.47	0.97	0.46	0.93	0.46	1.16	0.47	0.95	0.46	0.86	0.49	1.34
	8	0.0	0.0	0	0	0	0	0	0	0	0	0	0	0	0.011
						M_1 = 58,041						M_1 = 55,810			
72,165	1	0.637	1.0	0.63	1.0	0.60	1.0	0.65	1.0	0.63	1.0	0.55	1.0	0.66	1.0
	2	0.501	1.0	0.51	1.03	0.52	1.19	0.50	0.95	0.52	1.07	0.53	1.44	0.49	0.91
	4	0.462	1.0	0.46	1.03	0.48	1.44	0.46	0.93	0.46	1.05	0.51	2.20	0.46	0.86
	8	0.0	0.0	0	0	0	0	0	0	0	0	0.013	0.072	0	0
						M_1 = 61,160						M_1 = 62,260			
19,691	1	0.637	1.0	0.64	1.0	0.66	1.0	0.70	1.0	0.64	1.0	0.68	1.0	0.76	1.0
	2	0.501	1.0	0.50	1.0	0.50	0.92	0.44	0.73	0.50	1.0	0.49	0.85	0.83	0.53
	4	0.462	1.0	0.46	0.96	0.45	0.82	0.48	0.71	0.46	0.99	0.43	0.68	0.50	0.52
	8	0.0	0.0	0	0	0	0	0	0	0	0	0	0	0	0
						M_1 = 60,140						M_1 = 60,240			
135	1	0.637	1.0	0.64	1.0	0.64	1.0	0.66	1.0	0.64	1.0	0.65	1.0	0.68	1.0
	2	0.501	1.0	0.50	0.98	0.50	0.97	0.48	0.9	0.49	0.96	0.49	0.94	0.46	0.81
	4	0.462	1.0	0.46	0.99	0.46	0.96	0.47	0.9	0.46	0.97	0.46	0.94	0.48	0.82
	8	0.0	0.0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Argument determining random number set. [b] Data simulated with twice the usual standard deviation.

seen for the dimer in Table IV-D. In the latter case, the equilibrium cannot be distinguished from monomer–trimer and K_2 becomes meaningless. Both cases illustrate the expected loss in accuracy for K_i found even for a "good" numerical solution in terms of the analogous C_{i0} as the monomer concentration diminishes, since the latter enters the calculation as a divisor raised to a power. Although the LC calculations show some superiority, LTDC generally gives excellent values

TABLE VIII: Exponential Analysis of Self-Association. Effect on Solutions of Absolute Error in C.

				C Data Solutions				C Data Solutions				
		True Value		LC		LTDC		LC		LTDC		
Error in C	i	C_0	K	C_0	K	C_0	K	C_0	K	C_0	K	Error in C
						M_1 = 68,550				M_1 = 58,690		
	1	0.637	1.0	0.515	1.0	0.681	1.0	0.673	1.0	0.649	1.0	
−0.040	2	0.501	1.0	0.619	1.89	0.556	0.92	0.462	0.824	0.467	0.905	+0.01
	4	0.462	1.0	0.418	2.11	0.333	0.44	0.477	0.824	0.492	1.02	
	8	0.0	0.0	0	0	0	0	0	0	0	0	
						M_1 = 62,070				M_1 = 54,560		
	1	0.637	1.0	0.610	1.0	0.649	1.0	0.768	1.0	0.669	1.0	
−0.010	2	0.501	1.0	0.525	1.14	0.514	0.974	0.367	0.503	0.382	0.713	+0.04
	4	0.462	1.0	0.453	1.16	0.430	0.817	0.512	0.522	0.578	1.18	
	8	0.0	0.0	0	0	0	0	0	0	0.013	0.017	
						M_1 = 61,160						
	1	0.637	1.0	0.626	1.0	0.648	1.0					
−0.005	2	0.501	1.0	0.509	1.05	0.503	0.963					
	4	0.462	1.0	0.459	1.06	0.446	0.873					
	8	0.0	0.0	0	0	0	0					
								ΔC Data Solution				
						M_1 = 60,300				M_1 = 65,800		
	1	0.637	1.0	0.642	1.0	0.647	1.0	0.612	1.0	0.657	1.0	
0.0	2	0.501	1.0	0.494	0.969	0.492	0.948	0.522	1.13	0.551	0.995	
	4	0.462	1.0	0.465	0.974	0.462	0.927	0.455	1.16	0.369	0.608	
	8	0.0	0.0	0	0	0	0	0	0	0	0	
						M_1 = 59,476						
	1	0.637	1.0	0.657	1.0	0.648	1.0					
+0.005	2	0.501	1.0	0.478	0.893	0.480	0.93					
	4	0.462	1.0	0.471	0.895	0.477	0.98					
	8	0.0	0.0	0	0	0	0					

for the monomer molecular weight, as well as for the C_{i0} and K_i. An adequate solution is also obtained from ΔC data with the LC method but ΔC LTDC clearly has too great a choice in finding a good least-squares solution when neither C_m nor M_i is known.

Similar permutations of the K_i were made on the four-component monomer–dimer–tetramer–octamer system. The quality of the solutions was comparable with that obtained for the monomer–dimer–trimer system discussed above so that they are not presented in tabular form. One four-component case is shown later illustrating the effect of various errors. The more difficult monomer–dimer–trimer–tetramer system was successfully solved as part of a more complex association.

The five-component case was most extensively studied for the monomer–dimer–tetramer–hexamer–octamer (1–2–4–6–8) and 1–2–3–4–8 case (anticipated alternate dissociation modes of an eight-subunit enzyme with postulated D_4 symmetry under study in our laboratory (Haschemeyer, 1968)). Typical results are shown in Table V along with the single 1–2–3–4–5 case solved with all K_i = 1. Since only the LC method with absolute concentration data is capable of providing consistently reliable results with five solute components, the other solutions have not been included, even when acceptable.

The three five-solute cases considered each give satisfactory solutions when all K_i = 1 (Table V-A, -B, and -C). The mode of association is readily established when all components entering into association are adequately represented. These results are illustrated by the monomer–tetramer–octamer case solved as a 1–2–4–6–8 equilibrium system shown in Table V-D. Even if the K_i are chosen so that monomer and two other species are weakly represented (Table V-E) a reasonable solution is still obtained. Note that K_6 and K_8 are meaningless despite their large value when the C_{i0} are negligible. A 1–2–4–8 system with K_i selected to give octamer as the dominant species with dimer and tetramer weakly represented (Table V-F) resulted in alternating positive and negative coefficients characteristic of unsatisfactory solutions.

As already discussed, we have preliminarily extended the separation of exponentials method for self-associating systems to an algebraic solution for incompetence of multiple species in terms of a single species presumed totally competent. Results of 1–2–4 and 1–2–4–8 solutions are shown in Table VI for the monomer–dimer–tetramer case with 10% each of C_0 incompetent monomer and tetramer. The C_{im} data from simulated runs with C_0 = 1.6 and 0.8 were combined to yield corrected K_i.

TABLE IX: Exponential Analysis of Self-Association. Effect on Solutions of Error in M_1.

				LC Solutions				LC Solutions			
		True Value		Synthetic		Simulated		Synthetic		Simulated	
	i	C_0	K	C_0	K	C_0	K	C_0	K	C_0	K
M_1		60,000		44,000		44,000		62,000		62,000	
	1	0.64	1.0	0.37	1.0	0.37	1.0	0.67	1.0	0.68	1.0
	2	0.50	1.0	0.45	2.95	0.45	2.87	0.49	0.86	0.48	0.83
	4	0.46	1.0	0.69	19.6	0.68	18.8	0.44	0.72	0.44	0.70
	8	0.0	0.0	0.09	29.4	0.09	28.0	0	0	0	0
M_1		60,0000		54,000		54,000		66,000		66,000	
	1	0.64	1.0	0.53	1.0	0.53	1.0	0.75	1.0	0.76	1.0
	2	0.50	1.0	0.51	1.54	0.50	1.49	0.46	0.63	0.45	0.61
	4	0.46	1.0	0.53	2.87	0.54	2.77	0.40	0.38	0.40	0.38
	8	0.0	0.0	0.02	0.19	0.02	0.19	−0.013	0	0.013	0
M_1		60,000		58,000		58,000		86,000		86,000	
	1	0.64	1.0	0.60	1.0	0.60	1.0	1.15	1.0	1.16	1.0
	2	0.50	1.0	0.51	1.16	0.50	1.12	0.18	0.09	0.17	0.09
	4	0.46	1.0	0.49	1.41	0.49	1.37	0.30	0.03	0.31	0.03
	8	0.0	0.0	0	0.015	0	0.016	−0.04	0	−0.04	0
								LTDC Solution			
M_1		60,000		60,000		60,000		60,000		60,300	
	1	0.64	1.0	0.64	1.0	0.64	1.0	0.64	1.0	0.65	1.0
	2	0.50	1.0	0.50	1.0	0.49	0.97	0.50	1.0	0.49	0.95
	4	0.46	1.0	0.46	1.0	0.46	0.97	0.46	1.0	0.46	0.93
	8	0.0	0.0	0	0	0	0	0	0	0	0

Additional Error Consideration. The numerical solutions discussed thus far were all obtained on data with microcomparator reading error simulated from the same random number set. The effect of different random number sets, simulating repeated runs under identical conditions but with different random errors, was investigated for the monomer–dimer–tetramer case solved as a 1–2–4–8 system. Solutions were also obtained with the same random number sets incorporating a more generous error estimate corresponding to twice the standard deviation used in prior calculations. These results are given in Table VII. The first table entry corresponds to the random number set employed in the other tables for solution and may be characterized as "typical." A doubling of assumed error is mildly detrimental, but by no means disastrous. As might be anticipated, the quality of results with alternate methods of solution does not follow identical patterns with different random number sets.

Errors resulting from a sedimentation equilibrium experiment are not necessarily limited to reading of the plates. Proper alignment of the optical system and the attainment of base-line data which is neither sloping nor otherwise inconsistent with respect to the final equilibrium pattern (to within the 2 μ assumed) are by no means trivial considerations (Yphantis, 1964). A properly functional optical system is best ensured by extraordinary precautions at the time of alignment, for there unfortunately seems to be no simple *post facto* test for the degree of error expected in the final $C(X)$ *vs.* X^2 equilibrium curve due to faulty alignment procedures or optical components. Fortunately, it appears that with proper combination of experimental technique and cell components the base-line problem can be overcome, as may be confirmed experimentally by repeated measurements.

Error in the establishment of absolute concentration introduces a constant shift to correspondingly higher or lower values in $C(X)$ for all X^2 values. For the typical LaBar- (1965) type experiments described here, this error occurs in the determination of total meniscus concentration. Table VIII shows the effect of such errors on the solution of C data compared with the unique ΔC solution. It appears that when the error in absolute C exceeds 10 μ the ΔC method of solution may be preferred. For most systems where C_m can be determined by techniques similar to that described by Bethune and Simpson (1969) or Lewis *et al.* (1969), this error can be limited to 5 μ or less with relative ease.

It is assumed in the LC calculations that the monomer molecular weight was absolutely known. More typically this number will only be known to some approximation; the effect of such an error is shown in Table IX with 16 significant figure data analyzed alongside for comparison. As is seen, the values obtained for K from LC rapidly deteriorate with error in M_1, so that the LTDC solution becomes superior when the error in M_1 approaches 3%. The LTDC solutions are obtained most conveniently with estimates derived from the LC solution. It is encouraging to note that no convergence difficulties

were encountered when any of the LC solutions shown in Table IX were used as original estimates for LTDC.

Discussion

The direct numerical solution to the well-known exponential form of the concentration distribution expression for sedimentation equilibrium offers advantages in its simplicity and flexibility of application, and yields good results in the presence of realistic experimental error. The equations may be solved in many forms for almost any postulated limited discrete distribution. More complicated self-associating systems might be handled, for example, if the number of unknowns are reduced when some or all of the steps in the association may be described in terms of a single intrinsic equilibrium constant.

It seems inappropriate to attempt a full comparison here of the methods presented with others in current usage. Clearly the analysis of a few runs by the techniques discussed here should never be considered as a substitute for the beautifully thorough analysis performed in some of the references mentioned earlier. For many systems it is likely that the method of choice will depend on multiple factors including the accuracy with which molecular weights are already known, the availability of material, solubility, number of components present, possible presence of incompetence, span of molecular weights encountered, etc. For markedly nonideal systems, further extension of the treatment presented here is required.

The equations for self-associating systems may be written to include the second virial coefficient, B, in the exponents. The activity coefficient of the ith component, f_i, defined by $a_i = C_i f_i$, where a_i is the activity, is related to B at low concentrations by the equation $f_i = e^{iBMC}$ if all B are assumed equal (*e.g.*, Adams and Fujita, 1963; Van Holde *et al.*, 1968). The interference optical system measures

$$C(X) = \sum_i C_i(X)$$

while for nonideal components, the equilibrium distribution is given by

$$a_i(X) = a_{i\mathrm{m}} e^{iH(X^2 - X_\mathrm{m}^2)}$$

or

$$C_i(X) f_i(X) = C_{i\mathrm{m}} f_i(X_\mathrm{m}) e^{iH(X^2 - X_\mathrm{m}^2)}$$

The above expressions may be combined and rearranged to give the desired result

$$C(X) = \sum_i C_{i\mathrm{m}} e^{M[A(X^2 - X_\mathrm{m}^2) - B(C(X) - C_\mathrm{m})]}$$

where $A = H/M$ and the $C_{i\mathrm{m}}$ may be expressed as $C_{1\mathrm{m}}{}^i K_i$, if desired, since $f_i = f_1{}^i$. This equation is readily solved by LTDC, but is expected to decrease by one the number of species for which reasonable solution may be obtained due to the introduction of the additional unknown, B. The nonideal equation has not yet been tested for stability to experimental error and can therefore not be compared with other methods. It should be emphasized, however, that in a formal sense, all of the desired unknowns, B, M, and the K_i, may be directly evaluated.

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Isolation of γ-Tocotrienol Dimers from *Hevea* Latex*

C. K. Chow† and H. H. Draper

ABSTRACT: Two novel reducing compounds were isolated from *Hevea* latex lipid and shown to be dimers of γ-tocotrienol. They were obtained synthetically by oxidation of γ-tocotrienol with *p*-benzoquinone. Structural characterization indicated that the compounds are 5-(γ-tocotrienyloxy)-γ-tocotrienol and 5-(γ-tocotrienyl)-γ-tocotrienol.

Since the isolation of a dimer of α-tocopherol from mammalian tissues by Csallany and Draper (1963) the occurrence of other dimers of the tocopherols has been actively investigated. McHale and Green (1963) isolated a dimer of γ-tocopherol from cottonseed oil deodorizer scum and proposed that it was identical with a compound detected earlier by Shone (1963) in tung oil. They concluded that the compound was 5-(γ-tocopheryloxy)-γ-tocopherol (Figure 1, Ia). Komoda and Harada (1969) have reported a similar compound in soybean oil. In a study of the chemical oxidation products of various tocopherols, Nilsson *et al.* (1968) reported the synthesis of nine dimers and three trimers. Two dimers obtained by oxidation of γ-tocopherol with *p*-benzoquinone were identified as 5-(γ-tocopheryloxy)-γ-tocopherol and 5-(γ-tocopheryl)-γ-tocopherol (Figure 1, IIa). These two compounds also were found in corn oil.

Latex of the rubber tree *Hevea brasiliensis* contains an extraordinary concentration of tocopherols (about 8% of total lipids), mainly in the form of the unsaturated isomers (tocotrienols). During the isolation of these compounds for reference purposes, two novel reducing substances of low polarity were detected on thin-layer chromatograms. These compounds were isolated and found to be dimers of γ-tocotrienol. Evidence is presented that they are 5-(γ-tocotrienyloxy)-γ-tocotrienol (Figure 1, Ib) and 5-(γ-tocotrienyl)-γ-tocotrienol (Figure 1, IIb).

Experimental Section

Isolation of Compounds A and B. Samples (50 g) of *Hevea* latex were extracted with 750 ml of CH_3OH–$CHCl_3$ (1:2) according to the Folch *et al.* (1951) procedure. After being homogenized with a solvent in a Waring laboratory blendor for 3 min the samples were filtered and 0.2 volume of H_2O was added to the filtrate. The two solvent phases were allowed to separate overnight, whereupon the lower phase was evaporated to dryness under vacuum at 50° and the residue was taken up in diethyl ether. The ethereal solution was washed three times with water, dried over anhydrous sodium sulfate, and evaporated under N_2.

Approximately 700 mg of lipid was chromatographed on a 50 g Bio-Rad neutral alumina column deactivated with 6% water. The column was developed with 250-ml volumes of petroleum ether (twice-distilled Skellysolve F, bp 40–60°) containing the following proportions of peroxide-free diethyl ether: 0, 2, 4, 6, 8, 10, 12, 20, 40, and 100%. The residue from each fraction was chromatographed on thin layers of silica gel G using $CHCl_3$ as solvent. The plates were prepared with a 0.002% aqueous solution of 2′,7′-dichlorofluorescein. In addition to bands corresponding to standard tocopherols, two unidentified compounds were detected at R_F positions 0.9 and 0.8 by spraying with Emmerie–Engel reagent. These brownish yellow oils (A and B, respectively) were eluted with diethyl ether and purified by thin-layer chromatography on silica gel G until they gave a single spot in several systems. Isopropyl

* From the Division of Nutritional Biochemistry, Department of Animal Science, University of Illinois at Urbana-Champaign, Urbana, Illinois. *Received August 6, 1969.* This research was initiated by the second author while on leave at the Institute of Animal Physiology, Babraham, Cambridge, England.

† Present address is Department of Medicine, New York University Medical Center, New York, New York 10016.