The Determination of Density and Molecular Weight Distributions of Lipoproteins by Sedimentation Equilibrium

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SUMMARY

A method is presented by which an experimental record of total concentration as a function of radial distance, obtained in a sedimentation equilibrium experiment conducted with a noninteracting mixture in the absence of a density gradient, may be analyzed to obtain the unimodal distributions of molecular weight and of partial molar volume when these vary concomitantly and continuously. Particular attention is given to the characterization of classes of lipoproteins exhibiting Gaussian distributions of these quantities, although the analysis is applicable to other types of unimodal distribution. Equations are also formulated permitting the definition of the corresponding distributions of partial specific volume and of density. The analysis procedure is based on a method (employing Laplace transforms) developed previously, but differs from it in that it avoids the necessity of differentiating experimental results, which introduces error.

The method offers certain advantages over other procedures used to characterize and compare lipoprotein samples (exhibiting unimodal distributions) with regard to the duration of the experiment, economy of the sample, and, particularly, the ability to define in principle all of the relevant distributions from one sedimentation equilibrium experiment and an external measurement of the weight average partial specific volume. These points and the steps in the analysis procedure are illustrated with experimental results obtained in the sedimentation equilibrium of a sample of human serum low density lipoprotein. The experimental parameters (such as solution density, column height, and angular velocity) used in the conduction of these experiments were selected on the basis of computer-simulated examples, which are also presented. These provide a guide for other workers interested in characterizing lipoproteins of this class.

Each class of lipoprotein exhibits a continuous distribution of density and of molecular weight due to the large content of noncovalently bound lipid (1). Moreover, the distributions for LDL\(^1\) have been shown to vary with the physiological state of the individual (2-7). In view of this variability, it is desirable to establish methods for defining the characteristic distributions of a given sample without recourse to laborious fractionation techniques. Analytical ultracentrifugation experiments of two designs, analysis of boundary shapes in sedimentation velocity (8, 9) and sedimentation equilibrium in a buoyant density gradient (2-5), have been used to obtain such distributions. The former method suffers from the inherent disadvantage that boundary shapes are determined by factors other than heterogeneity with respect to molecular weight and density, whereas the latter requires subjecting the sample to long centrifugation times in a medium of high salt and sucrose concentration. Moreover, because density heterogeneity broadens the sedimentation equilibrium concentration distribution in a buoyant density gradient, the apparent mean molecular weight is lowered (2-5, 10), necessitating separate experiments for molecular weight estimation.

The present work examines the potential of analyzing sedimentation equilibrium results obtained in the absence of a density gradient in terms of both density and molecular weight distributions of low density lipoprotein. The analysis procedure is based on that proposed by Donnelly (11, 12), but differs from it in the following ways: the concomitant variation of both molecular weight and partial molar volume is explicitly considered; an alternative function capable of inverse Laplace transformation is presented which permits the direct analysis of experimental results without the need of recasting them in differential form; and the problem is considered in relation to equations describing Gaussian distributions appropriate to certain lipoproteins, thereby permitting the formulation of expressions to obtain distributions of molecular weight, partial molar volume, partial specific volume, and density. In addition, numerical simulations of sedimentation equilibrium experiments are presented which permit comment on the optimum choice of experimental parameters. Finally, the method and its limitations are illustrated with results obtained with a sample of human serum low density lipoprotein.

THEORY

Basic Relations—The sedimentation equilibrium distribution of each solute \(i\) in a mixture is described in terms of weight concentration by (13, 14),

\[ c_i(t) = c_i(0) e^{-rt} \]

where \(c_i(t)\) is the concentration of solute \(i\) at time \(t\), \(c_i(0)\) is the initial concentration, and \(r\) is the rate constant for the process. The rate constant is related to the apparent sedimentation coefficient \(S_{app}\) by

\[ r = \frac{1}{S_{app}} \]

and the sedimentation coefficient is related to the diffusion coefficient \(D\) by

\[ S = \frac{r}{D} \]

The sedimentation coefficient is also related to the partial specific volume \(\bar{v}_i\) and the partial specific volume is related to the fractional change in volume of the solute when it is added to the solvent by

\[ \bar{v}_i = \frac{v_i}{M_i} - 1 \]

where \(v_i\) is the specific volume of the solute and \(M_i\) is the molecular weight of the solute.

Furthermore, the sedimentation equilibrium distribution is related to the partial specific volume by

\[ c_i(t) = \frac{c_i(0)}{1 + \frac{t}{S_{app}}} \]

and the partial specific volume is related to the refractive index increment \(dn/dc\) by

\[ \bar{v}_i = \frac{dn_i}{dc_i} \]

where \(dn_i/dc_i\) is the refractive index increment of the solute.

Finally, the sedimentation equilibrium distribution is related to the molecular weight by

\[ c_i(t) = \frac{c_i(0)}{1 + \frac{t}{D_i}} \]

and the molecular weight is related to the diffusion coefficient by

\[ D_i = \frac{1}{S_i} \]

where \(S_i\) is the sedimentation coefficient of the solute.

These relations provide a framework for the analysis of sedimentation equilibrium experiments and can be used to obtain the unimodal distributions of molecular weight and of partial molar volume when these vary concomitantly and continuously.

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1 The abbreviation used is: LDL, serum low density lipoprotein.
where $r$ and $r_1$ are any radial distances between or within the meniscus and base positions, respectively; $M_i$ is the molecular weight of species $i$ with partial specific volume $v_i$; $\omega$ is the angular velocity, $\rho$ the solution density, $R$ the gas constant, and $T$ the absolute temperature. It is implicit in Equation 1 that the activity coefficient of each species is unity. It will also be assumed that $v_i$ and $\rho$ are constants independent of radial position.

For a nonreacting mixture, the amount in grams of assumed that $p_i$ and $p$ are constants independent of radial activity $o$ of each species is unity. It will also be velocity, $p$ the solution density, $R$ the gas constant, and $T$ the absolute temperature. It is implicit in Equation 1 that the weight of species $i$ with partial specific volume $v_i$; $w$ is the angular

Equation 3 becomes

When summed over all of the species in the nonreacting mixture,

Combination of Equations 1 and 2 yields

\[
Q_i = c_i(r_1)\theta(1 - \omega^2(1 - \theta)(\rho) / 2RT) \]

(1b)

where $\theta$ and $h$ are respectively the cell sector angle and thickness. Combination of Equations 1 and 2 yields

\[
c_i(r) = 2h_i M_i Q_i / \theta y_i \]

(3a)

\[
y_i = \phi_i M_i (r_i^2 - r^2) / \phi_i M_i (r_i^2 - r_{i-1}^2) \]

(3b)

When summed over all of the species in the nonreacting mixture, Equation 3 becomes

\[
\bar{\xi}(r) = \sum c_i(r) - (2 \sum (\theta_i M_i Q_i / y_i)) / \theta \]

(4a)

or,

\[
d \ln \bar{\xi}(r) / dr^2 = \sum (\theta_i M_i Q_i / y_i) / \sum (\theta_i M_i Q_i / y_i) \]

(4b)

Equation 4a describes the distribution of the total solute concentration with radial distance in terms of the amounts of each species present in the original solution. It may be rewritten in terms of dimensionless parameters as follows (15). Equation 1 is reformulated as,

\[
c_i(\xi) = c_i(r_0) e^{-v_i M_i \xi} \]

(5a)

\[
A_i = \left( r_{i-1}^2 - r_i^2 \right) / r_{i-1} h \]

(5b)

\[
\xi = \left( r_i^2 - r^2 \right) / b \]

(5c)

Combination of Equations 2 and 5 yields (on noting that $Q_i = c_{0,i} V_{cell} = c_{0,i} 0 h b / 2$)

\[
c_i(\xi) = A_i M_i c_{0,i} e^{-v_i M_i \xi} / (1 - e^{-v_i M_i \xi}) \]

(6)

where $c_{0,i}$ is the initial weight concentration of species $i$. Division of Equation 6 by $c_{0,i}$ the total initial concentration, gives

\[
c_i(\xi) / c_{0,i} = A_i M_i f e^{-v_i M_i \xi} / (1 - e^{-v_i M_i \xi}) \]

(7)

where $f_i$ is the weight fraction of $i$. Summation of Equation 7 gives

\[
\theta(\xi) = \bar{\xi}(\xi) / c_{0,i} = \sum \left( A_i M_i f e^{-v_i M_i \xi} / (1 - e^{-v_i M_i \xi}) \right) \]

(8)

Gaussian Distributions—For systems comprising a population of noninteracting species with a Gaussian distribution of molecular weight about the mean, $M$, the continuous distribution is now visualized as a mixture of $n$ species of $\bar{V}_i$ given by Equation 13 and of the relative amount given by the product of $10g_i / n$ and the right-hand side of Equation 14. In these terms

\[
Q_i = 10Q_i / n \sqrt{2\pi e^{3/2(n-1)g_i} / \bar{M}_i} \]

(15)

The same equation follows by analogous reasoning based on Equation 9. For a distribution of sedimentation equilibrium data, $F(\bar{V}_i)$ in units of partial specific volume about the mean $M$, and $\bar{F} = \bar{F}_i$ is a constant. The above formulation being consistent with the relation $f(M_i) dM_i = F(\bar{V}_i) d\bar{V}_i$. Entirely similar relations are used to relate other distribution functions which follow.
8 could be expressed as a Laplace transform. Thus, for continuous distributions where neither $M_i$ nor $V_i$ assume negative values, Equation 8 may be written as

$$\theta(t) = \int_0^\infty L(tg(t_i)e^{-t_i}(1-e^{-t_i})dt_i = L(tg(t_i)/(1-e^{-t_i}))$$

where $L$ denotes the Laplace transform operator and $t_i = \Lambda_iM_i$. For the system described by Equations 9, 10, and 11,

$$t_i = (\bar{M}_i\bar{V} - \bar{V} + \bar{V}_i)/(1 - \bar{\theta}_p)\omega^b/2RT\bar{V}$$

It follows that

$$g(t_i) = 2RT\bar{V}F(\bar{V}_i)/(1 - \bar{\theta}_p)\omega^b$$

or with the use of Equation 11,

$$g(t_i) = 1/\sqrt{2\pi}(\bar{V}_i - \bar{V})$$

where $\bar{t}$ is identified as the value of $t_i$ when $\bar{V}_i = \bar{V}$. Equation 16 may be inverted to give,

$$g(t_i) = (1 - e^{-\bar{t}_i})L^{-1}(\theta(t_i))/\bar{t}_i$$

It follows that $g(t_i)$ may only be written as an explicit function of $t_i$ if a function, capable of inversion, may be found which describes the experimentally available plot of $\theta(t_i)$ versus $t_i$. Later, a function will be presented which serves this purpose directly; but for the present it is convenient to summarize briefly the approach outlined by Donnelly (11, 12). He suggested that a plot be constructed of $F$ versus $u$, where $F = 1/d \ln \left( r_i/d(r_i) \right)$ and $u = 1 - \xi$. In the cases where this plot proves to be curvilinear, an appropriate relation describing it is,

$$F = (P - Qu)/(1 + R(P - Qu))$$

where $P, Q, and R$ are constants determinable by least square regression. The function $F$ is related to $\theta(t_i)$ by,

$$\theta(t_i) = \exp\int_0^{b/2} \frac{du}{F}$$

whereupon it follows directly that

$$L^{-1}(\theta(t_i)) = \frac{\xi(t_i)(P/Q)^{1/2}t_i}{c_0\Gamma(b/Q)}$$

where $\Gamma(b/Q)$ is the gamma function of $b/Q$. A mathematical requirement for the applicability of Equation 22 is that $t_i > bR$. The essence of the Donnelly method then is to employ experimentally determined values of $\xi(t_i), c_0, P, Q, and R$ to construct a plot of $g(t_i)$ versus $t_i$ with the combined use of Equations 19 and 22.

For systems described by Equations 9, 10, and 11, not discussed by Donnelly, the plot of $g(t_i)$ versus $t_i$ will be Gaussian according to Equation 18b and the remaining problem is to transform this plot to corresponding plots of $F(\bar{V}_i)$ versus $\bar{V}_i$ and $f(M_i)$ versus $M_i$. One approach is to note that differentiation of Equation 18b with respect to $t_i$ establishes that the turning point occurs at a value of $t_i = \bar{t}_i$. Because $\bar{t}_i$ has been identified as the value of $t_i$ when $\bar{V}_i = \bar{V}$, Equation 17a may be written as

$$\bar{M} = 2RT\bar{V}/(1 - \bar{\theta}_p)\omega^b$$

where $\bar{v}$ is the partial specific volume of the species whose partial molar volume is $\bar{V}$. Because $\bar{v}$ closely approximates the weight average partial specific volume available from independent density measurements, Equation 23 may be used to estimate $\bar{M}$. It is now possible to transform the $g(t_i)$ versus $t_i$ plot to one of $F(\bar{V}_i)$ versus $\bar{V}_i$ (using Equations 17a and 18a) and to one of $f(M_i)$ versus $M_i$ (using Equations 10 and 12). Each of these plots will be Gaussian, whereas those of $f(\bar{V}_i)$ versus $\bar{t}_i$, and of $G(\bar{V}_i)$ versus $\bar{V}_i$, will not. The latter distributions may be found using the following relations,

$$\bar{t}_i = \bar{V}_i\bar{\theta}_p/(\bar{M}_i\bar{V} - \bar{V}_i)$$

$$f(\bar{V}_i) = F(\bar{V}_i)(\bar{M}_i\bar{V} - \bar{V}_i)/(2RT\bar{V})$$

$$G(\bar{V}_i) = -f(\bar{V}_i)\bar{v}_i$$

In Equation 24c, $\rho_i$ is the density of solute species $i$ assumed to equal $1/\bar{v}_i$.

Two basic questions remain in relation to the possible application of the theory to the elucidation of real lipoprotein systems. The first concerns the relevance of Equation 20 in describing plots of $F$ versus $u$; the second, the feasibility of designing experiments which will permit the evaluation of the required parameters. Both questions are explored in the next section with the aid of numerical examples.

**NUMERICAL ILLUSTRATIONS**

Fig. 1A presents a plot of $F$ versus $u$ calculated using Equations 3b, 4b, 10, 13, and 15 for a system exhibiting Gaussian distribution. The sedimentation parameters were as follows: angular velocity, 8000 rpm; column height, 0.15 cm; solution density, 1.030 g per ml; initial concentration, 5 g per liter, and temperature, 293.16 K. A, plot of $F = 1/d \ln r_i/d(r_i)$ and $u = 1 - \xi$. In the cases where this plot proves to be curvilinear, an appropriate relation describing it is,

$$F = (P - Qu)/(1 + R(P - Qu))$$

where $P, Q, and R$ are constants determinable by least square regression. The function $F$ is related to $\theta(t_i)$ by,

$$\theta(t_i) = \exp\int_0^{b/2} \frac{du}{F}$$

whereupon it follows directly that

$$L^{-1}(\theta(t_i)) = \frac{\xi(t_i)(P/Q)^{1/2}t_i}{c_0\Gamma(b/Q)}$$

where $\Gamma(b/Q)$ is the gamma function of $b/Q$. A mathematical requirement for the applicability of Equation 22 is that $t_i > bR$. The essence of the Donnelly method then is to employ experimentally determined values of $\xi(t_i), c_0, P, Q, and R$ to construct a plot of $g(t_i)$ versus $t_i$ with the combined use of Equations 19 and 22.

For systems described by Equations 9, 10, and 11, not discussed by Donnelly, the plot of $g(t_i)$ versus $t_i$ will be Gaussian according to Equation 18b and the remaining problem is to transform this plot to corresponding plots of $F(\bar{V}_i)$ versus $\bar{V}_i$ and $f(M_i)$ versus $M_i$. One approach is to note that differentiation of Equation 18b with respect to $t_i$ establishes that the turning point occurs at a value of $t_i = \bar{t}_i$. Because $\bar{t}_i$ has been identified as the value of $t_i$ when $\bar{V}_i = \bar{V}$, Equation 17a may be written as

$$\bar{M} = 2RT\bar{V}/(1 - \bar{\theta}_p)\omega^b$$

where $\bar{v}$ is the partial specific volume of the species whose partial molar volume is $\bar{V}$. Because $\bar{v}$ closely approximates the weight average partial specific volume available from independent density measurements, Equation 23 may be used to estimate $\bar{M}$. It is now possible to transform the $g(t_i)$ versus $t_i$ plot to one of $F(\bar{V}_i)$ versus $\bar{V}_i$ (using Equations 17a and 18a) and to one of $f(M_i)$ versus $M_i$ (using Equations 10 and 12). Each of these plots will be Gaussian, whereas those of $f(\bar{V}_i)$ versus $\bar{t}_i$, and of $G(\bar{V}_i)$ versus $\bar{V}_i$, will not. The latter distributions may be found using the following relations,

$$\bar{t}_i = \bar{V}_i\bar{\theta}_p/(\bar{M}_i\bar{V} - \bar{V}_i)$$

$$f(\bar{V}_i) = F(\bar{V}_i)(\bar{M}_i\bar{V} - \bar{V}_i)/(2RT\bar{V})$$

$$G(\bar{V}_i) = -f(\bar{V}_i)\bar{v}_i$$

In Equation 24c, $\rho_i$ is the density of solute species $i$ assumed to equal $1/\bar{v}_i$.

Two basic questions remain in relation to the possible application of the theory to the elucidation of real lipoprotein systems. The first concerns the relevance of Equation 20 in describing plots of $F$ versus $u$; the second, the feasibility of designing experiments which will permit the evaluation of the required parameters. Both questions are explored in the next section with the aid of numerical examples.

**NUMERICAL ILLUSTRATIONS**

Fig. 1A presents a plot of $F$ versus $u$ calculated using Equations 3b, 4b, 10, 13, and 15 for a system exhibiting Gaussian distribution.
The angular velocity for the system under discussion is 8,000 rpm where

$$v = 2.1142 \times 10^6, \quad \sigma_P = 0.4 \times 10^6$$

and of $M_1$ ($M = 2.2 \times 10^6, \sigma_M = 0.3884 \times 10^6$): these values are in realistic ranges for human low density lipoprotein where the variable lipid component has a weight average partial specific volume of 1.03. In the evaluation of $F$ by the summation method, 50 intervals ($n = 50$) were used because larger values of $n$ led to no significant improvement in the estimates. The plot in Fig. 1A is evidently curvilinear and is fitted well by Equation 20 as is shown by the solid line, calculated with values of $P = 2.4221 \times 10^3, Q = 1.3579 \times 10^4$, and $R = -4.1208 \times 10^6$ computed using Equation A10 of Donnelly (11) corrected for minor errors. These values were employed in Equations 19 and 22, together with a value of $e(r_{nl}) = 3.7074$ calculated with $n = 50$ from Equations 3, 10, 13, and 15 to compute a plot of $g(t)$ versus $t$ which is shown by the circles in Fig. 1B. In the calculation of $g(t)$, a value of $b/Q$ of 1.5299 × 101 was encountered and accordingly the gamma function of this quantity was replaced by Stirling's approximation thereby permitting a collection of terms in Equation 22 and their logarithmic evaluation with the aid of a computer. In relation to Fig. 1B it could be noted first that it is Gaussian in form and second that a negligible proportion of the distribution falls outside the limit dictated by the requirement that $t_i > bR = -8.5734 \times 10^6$. The solid line in Fig. 1B was computed on the basis of the selected distribution described by Equation 11 and with the use of Equations 17a and 18a. Clearly, the method of analysis based on Equation 20 has succeeded in reproducing the original distribution.

Similar calculations were performed with the same parameters describing the lipoprotein system as used in Fig. 1, but varying systematically $\omega, b$, and $r$. In all of the cases studied, plots of $F$ versus $u$ were curvilinear and describable by values of $P, Q$, and $R$ (Equation 20) which in turn lead to Gaussian distributions of $t_i, M_i$, and $V_i$ in agreement with those originally selected. In spite of the success of these numerical examples, in the analysis of actual experimental results at least two other factors must be considered. First, the concentration at the meniscus must be measurable with sufficient precision because it appears directly in Equation 22; this implies that with a refractometric optical system the fringe density at the cell base (or its equivalent for schlieren optics) must also be measurable. Even with an absorption optical system a sufficient amount of the distribution must be observable to enable the curvilinearity of the plot of $F$ versus $u$ to be defined. The second factor pertains to the extent of this curvilinearity, which is conveniently defined as the maximum percentage deviation of the $F$ versus $u$ curve from a straight line joining the points where $u = 0$ and 1. These factors are explored in Table I. The first three rows of Table I illustrate the effect of changing $\omega$ and it is clear from the last column that the curvilinearity is markedly increased as $\omega$ increases, of this set only 0.8% being experimentally undetectable. On the other hand, as $\omega$ increases, the base concentration gradient increases to a level at $\omega = 12,000$ rpm corresponding to 550 fringes per cm (assuming $h = 1.2$ cm and a constant specific refractive increment of $1.54 \times 10^{-4}$ liters per g for all of the lipoprotein species) outside the accepted measurable level with conventional interference optics of 200 fringes per cm (16). This does not exclude the possibility that the experiment could be conducted at this speed employing schlieren optics and a commercially available cell of shorter light path; but this would necessitate the integration of the experimental record to find $e(r)$ at each $r$. It therefore appears that the optimum angular velocity for the system under discussion is 8,000 rpm where detectable curvilinearity and measurable fringe density at the base (150 fringes per cm) is obtained. Moreover, for this $\omega$, the value of $e(r_{nl})$, which is greater than the value (3.5122 g per liter) predicted from Equation 3 for a single solute of $M = 2.2 \times 10^6$ and $V = 2.1142 \times 10^6$, may also be obtained readily. Comparison of Rows 2 and 4 of Table I shows the effect of doubling the column height. Clearly, increasing the column height offers the advantage of improving the curvilinearity but with the associated disadvantages of increased gradients near the base of the cell and considerably increased time required to reach equilibrium. Finally, a comparison of Rows 2, 5, and 6 illustrates the effect of changing the solution density. This is evidently a critical factor because as $\rho$ decreases from 1.03 to 1.00 the curvilinearity decreases from 17% to the undetectable level of 0.05% and at the same time the gradient at the base increases from a value of 150 fringes per cm to a nonmeasurable level of 820 fringes per cm. It may be concluded from Table I that it appears feasible to construct a plot of $g(t)$ versus $t$ from an experimental record obtained with suitably selected parameters and indeed Fig. 1, based on the values given in Row 2 of Table I, illustrates this. At the same time, it may also be concluded that an experiment conducted at low values of $\omega$ and $\mu$ may well lead to a plot of $F$ versus $u$ indistinguishable from that of a single solute ($F$ almost being constant).

A question not yet examined is the effect of selecting a solution density close to or at the density corresponding to the measurable weight average partial specific volume. The trend discussed in relation to Rows 2, 5, and 6 of Table I might at first sight suggest that this was a favorable condition for the conduction of a sedimentation equilibrium experiment. However, numerical examples have shown that with this selection of solution density, plots of $e(r)$ versus $r$ exhibit a shallow minimum and accordingly that plots of $F$ versus $u$ are discontinuous, an example being shown by the circles in Fig. 2 calculated with all of the parameters as in Fig. 1 except the value of $\rho$ which was set equal to 1.041. The solid line in Fig. 2 was computed on the basis of Equation 20 employing the values of $P, Q$, and $K$ computed as before (11). It is seen that Equation 20 no longer describes the data, particularly in the region of the asymptote. It therefore appears undesirable to select $\rho$ as the reciprocal measured partial specific volume and this view is reinforced by the possibility that such a system may tend to be gravitationally unstable.

To this point only one value of $\sigma_P$ (and hence of $\sigma_M$) has been
considered and yet the potential of the method may well reside in the ability to compare systems with different standard deviations. Accordingly, systems Gaussian in partial molar volume \( V \) with standard deviations, \( \sigma_V = 0.2 \times 10^5 \), 0.4 \( \times 10^5 \), and 0.6 \( \times 10^5 \) were examined numerically using \( F = 2.2 \times 10^4 \) and \( F = 1.03 \); the sedimentation equilibrium parameters were as reported in Row 2 of Table 1. The range of \( \sigma_F \) examined encompasses that encountered with low density lipoprotein systems. In each case, the plot of \( F \) versus \( u \) was curvilinear as exemplified by Fig. 1A. The curvilinearity increased with the increasing value of \( \sigma_F \) (the fringe density at the cell base decreasing). Moreover, in each case, distinguishable plots of \( g(t_i) \) versus \( t_i \) were obtained in agreement with the selected distributions. Evidently, the analysis procedure is applicable in each case, although it was noted when \( \sigma_F \) increased to the (perhaps unrealistic) value of \( 0.7 \times 10^6 \) and \( \rho \) maintained at \( 1.03 \) that behavior similar to that shown in Fig. 2 was obtained.

In summary, although it has not been possible to explore the complicated interrelationships between all of the variables, \( \omega, \rho, b, \) and \( c_0 \), it is clear that conditions may be found which on the one hand distinguish the sedimentation equilibrium behavior of a lipoprotein system from that of a single solute, and on the other hand result in a continuous plot of \( F \) versus \( u \) describable by Equation 20. Although the values cited in Table 1 provide a guide to an experimenter working with low density lipoproteins, it may be necessary to perform a series of experiments (particularly employing different solution densities away from the mean) in order to obtain a sedimentation equilibrium distribution amenable to analysis. This offers little problem in view of the availability of multi-channel cells and multi-place rotors.

**Preparation of LDL**—LDL was prepared from the 1-month-old plasma of a normal blood donor by precipitating with 10\% dextran sulfate (2 ml/100 ml of plasma) plus 1 ml CaCl\(_2\) (10 ml/100 ml of plasma). The precipitate was collected by centrifugation, dissolved in 2 ml NaCl (2.5 ml/100 ml of original plasma), and centrifuged in a Spinco model L ultracentrifuge to obtain the LDL as a middle orange layer (17). The sample was further purified by successive ultracentrifugation at solution densities of 1.063 g per ml, 1.019 g per ml, and again at 1.063 g per ml (18). All of the solvents were prepared as previously described (19) with the addition of EDTA-Na\(_2\) (0.5 g per liter) and adjustment to pH 7.4. The sample was initially analyzed by subjecting it to sedimentation velocity; the sedimentation equilibrium experiment which followed was completed within 48 hours after final purification.

**RESULTS AND DISCUSSION**

Fig. 3 presents a schlieren pattern obtained in the sedimentation velocity of the LDL sample, from which it is clear that the sample is free of any contaminant and that the distribution is unimodal. The result obtained in the sedimentation equilibrium of this sample conducted at 8000 rpm is shown in Fig. 4A as a plot of \( c(r) \) versus \( r \) and in Fig. 4B as a plot of \( F \) versus \( u \). The solid line in Fig. 4B was calculated using Equation 20 and the values \( F = -5.3843, Q = 9.5167, \) and \( R = 0.1741 \), obtained in an analysis of the data using the corrected Equation A10a of Donnelly (11). When \( c(r) \) versus \( r \) was employed, it was found that the resulting curve exhibited no turning point. Associated with this failure to define a distribution is the observation that the requirement for the applicability of Equation 22, \( t_i > \delta t_i = 0.2747 \) could not be met for any realistic LDL system. A possible cause of this failure is the error introduced in the determination of \( F \) by a differentiation procedure. Although the situation may be improved by taking readings at closer intervals of \( r \), it is preferable in principle to avoid the necessity of differentiating the results at all. This problem, of course, does not arise in the construction of numerical examples aimed at experimental design where the concept of the curvearity of the \( F \) versus \( u \) plot retains its usefulness.

In fact, it is possible to avoid the necessity of differentiating by noting that Equation 19 merely requires the specification of
FIG. 3. A schlieren pattern recorded in the sedimentation velocity of the human serum LDL sample, conducted at an angular velocity of 52,000 rpm and at 17.4° with phase-plate angle 70°. The solution (7.906 g per liter) was initially dialyzed against a solvent of density 1.00473 g per ml and pH 7.4, the dialysate being used in the reference channel of the double sector cell. The pattern (sedimentation from left to right) was recorded 26 min after attaining maximum speed.

a function which is capable of inverse Laplace transformation and describes the \( \tilde{e}(r) \) versus \( r \) result obtained directly in the experiment. It may be shown that the function given in the following Equation 25 serves this purpose

\[
\theta(t) = \alpha_1 e^{\alpha_2 t} / (\xi - \alpha_2)^{\alpha_4}
\]

(25)

where \( \alpha_1, \alpha_2, \alpha_3, \) and \( \alpha_4 \) are constants. With the use of the second translation property of Laplace transforms, the invert of Equation 25 becomes

\[
\mathcal{L}^{-1}\{\theta(t)\} = \frac{\alpha_1 (i_1 + \alpha_2)^{i_3}(\xi - \alpha_2)^{i_4}}{\Gamma(\alpha_2)}; \quad i_1 > -\alpha_2
\]

(26)

where the symbol \( \Gamma \) again denotes a gamma function. Direct comparison of Equations 22 and 26 permits the following identifications,

\[
\begin{align*}
\alpha_1 &= \mathcal{E}(\tau_m)(P/Q)^{\alpha_2}\rho^{\alpha_2}; \quad \xi_0 \\
\alpha_2 &= -bR \\
\alpha_3 &= -((P/Q) - 1) \\
\alpha_4 &= b/Q
\end{align*}
\]

(27)

This shows that the use of Equation 25 is entirely equivalent to the use of Equation 20, with the advantage that the \( \tilde{e}(r) \) versus \( r \) result can be fitted directly. This may be seen more clearly by rewriting Equation 25 using Equation 27 to write \( \alpha_1 \) in terms of \( \alpha_0, \alpha_2, \) and \( \alpha_4 \).

Equation 25 becomes

\[
\tilde{e}(r) = \left( \frac{1 - \alpha_0}{\xi - \alpha_2} \right)^{\alpha_4} e^{\alpha_2 t} - t^\alpha
\]

(28)

Once \( \alpha_0, \alpha_2, \) and \( \alpha_4 \) (and hence \( \alpha_1 \)) have been determined, Equations 19 and 28 may be used to construct a plot of \( g(t_i) \) versus \( t_i \). It is recognized that it is extremely difficult to obtain a unique set of values of \( \alpha_0, \alpha_2, \) and \( \alpha_4 \) which describes, according to Equation 28, experimental data of the type shown in Fig. 4A and indeed attempts to find a satisfactory minimization procedure for this purpose were unsuccessful. In order to proceed, therefore, it is necessary to invoke the step function requirement that \( t_i \) be greater than \(-\alpha_2 \) (Equation 26) together with the physical information that for real LDL systems \( t_i \) spans a relatively narrow range of values which can be estimated to a first approximation for a particular system \( t_i = 10/(1 - \rho^2) \times 10^5 \). These observations are used in the following way. With \( Z = e(r)/\tilde{e}(r_m) \), Equation 28 may be rearranged to give, for two points on the experimental \( \tilde{e}(r) \) versus \( r \) plot,

\[
\begin{align*}
\alpha_0 &= \frac{Z_i}{(1 - \rho^2)^{\alpha_4}/(\alpha_2/\alpha_0)}^{1/1} \\
\alpha_2 &= \ln((1 - \alpha_2)(\rho^2 - 1)^\alpha_4/\alpha_0) \\
\alpha_3 &= \ln((1 - \alpha_2)(\rho^2 - 1)^\alpha_4/\alpha_0)
\end{align*}
\]

(29)

\( Z \) Both the methods of Fletcher and Dowell and Marquardt were tested by assigning exact values to \( \alpha_0, \alpha_2, \) and \( \alpha_4 \) to generate theoretical data free of experimental error using Equation 28. It was found that both methods failed to return the assigned values from the simulated data.
The two pairs of values \( \xi_1, Z_1 \) and \( \xi_2, Z_2 \) are chosen to be sufficiently separated to indicate the trend of the data without introducing errors associated with the measurements made close to the meniscus or cell base. The constants \( \alpha_2 \) and \( \alpha_4 \) are then evaluated from Equations 29 for fixed values of \( \alpha_3 \). In the present work, \( \alpha_2 \) and \( \alpha_4 \) were calculated for values of \( \alpha_3 \) from 0 to \(-10,000 \) in increments of 50 to ensure that a sufficiently wide range was investigated. It was found that of the 200 sets of values of \( \alpha_2, \alpha_3, \) and \( \alpha_4 \) obtained in this way, all except those corresponding to \( \alpha_3 \) in the range \(-50 \) to \(-300 \), could be eliminated by the requirement mentioned above that \( t_i \) be greater than \(-\alpha_2 \). The small domain of permitted values of \( \alpha_3 \) thus discovered is then searched at smaller intervals and the complete \( c(r) \) versus \( r \) curve is generated from each set of values of \( \alpha_2, \alpha_3, \) and \( \alpha_4 \) by Equation 28. The process is continued until the calculated concentration distribution differs from that experimentally determined by less than the experimental error of measurement, over the entire range from the meniscus to the cell base. This procedure leads to different sets of \( \alpha_2, \alpha_3, \) and \( \alpha_4 \), all describing the \( c(r) \) versus \( r \) plot within experimental error, but leading to very similar plots of \( g(t_i) \) versus \( t_i \) calculated from Equations 19 and 26. The solid points shown in Fig. 5A were computed with values of \( \alpha_2 = 18.3297, \alpha_3 = -100.99, \) and \( \alpha_4 = 1871.58 \), of which the set examined best fitted the \( c(r) \) versus \( r \) plot. The solid points in Fig. 5A suggest a Gaussian distribution with the maximum occurring at a value of \( t_1 = \bar{t} = 0.090 \). Accordingly, Equation 18b with \( \bar{t} = 0.090 \) and varying values of \( \sigma_2 \), was employed to fit the experimental data with the aid of a computer and a Hewlett Packard 7200 A graphic plotter until the value of \( \sigma_2 \), giving the best fit was obtained. The result, shown as a solid line in Fig. 5A indicates that the \( g(t_i) \) distribution for the LDL sample closely approximates Gaussian in form with standard deviation \( \sigma_1 = -0.42 \).

Earlier in this work, transforms were presented which allowed the distributions in partial molar volume, molecular weight, partial specific volume, and density to be derived from a Gaussian distribution of \( g(t_i) \). In order for such transforms to be applied, it is required that at least one value of molecular weight encompassed by the distribution, and the corresponding partial specific volume, be known. Three possible approaches to the determination of such reference values may be considered.

1. The apoprotein may be taken as a reference with a molecular weight of 500,000 (22) and a partial specific volume of 0.73 ml per g. Application of Equations 17a and 18a with \( \bar{v}_F = 1.03 \) (2, 6) would then allow transformations from \( g(t_i) \) versus \( t_i \) to \( F(V_i) \) versus \( V_i \), the distributions in molecular weight, partial specific volume, and density following from Equations 10, 12, and 24. The disadvantages of choosing such a reference are the uncertainties in the assignment of a molecular weight, and partial specific volume to the apoprotein and more importantly the use of \( \bar{v}_F = 1.03 \) which although applicable to the variable lipid component of LDL (2, 6) may not be applicable to the total lipid.

2. The mean molecular weight of the distribution, \( \bar{M} \), may be evaluated from Equation 23. The merit of this method is that all of the required information is available from one sedimentation equilibrium experiment and one associated partial specific volume determination. However, a practical limitation is the high sensitivity of the value of \( \bar{M} \) to errors in the partial specific volume because of the requirement (Table I) that the sedimentation equilibrium experiment be performed at a solvent density not far removed from that of the mean of the distribution. In the present work, an apparent value of \( \bar{M} \) was measured to be 0.963 ml per g and at the solvent density 1.03212 g per ml used in the sedimentation equilibrium experiment, the estimated error of \( \pm 0.004 \) ml per g in \( \bar{M} \) leads to estimates of \( \bar{M} \) which differ by a factor of 5. Acceptable precision in the molecular weight determined in this way therefore would require the accurate determination of the partial specific volume to at least four decimal places. It is also relevant in this connection to point out that the weight average partial specific volume which can be measured experimentally is slightly different from the theoretical average corresponding to \( M \) both because \( \bar{M} \) is strictly a number average quantity and because a Gaussian distribution of molecular weights is associated with a slightly non-Gaussian distribution of partial specific volume, as noted earlier. In the example (Fig. 1) derived from numerical simulation, the difference in the weight average partial specific volume and that required by the theory was only 0.006% but even this difference may become significant when sedimentation equilibrium experiments are performed at solvent densities leading to very low values of the \((1 - \bar{M})\) term.

3. A third method, free of the objections discussed under 1 and 2, requires an independent evaluation of the mean molecular weight of the sample. Schumaker and Adams (2) have pointed out that at solution densities sufficiently far removed from that of the mean of the distribution, the sedimentation behavior of an LDL sample approximates that of a single solute, and they have made use of this property to evaluate molecular weights from the flotation velocity experiments conducted at high solution densities. In the present analysis, the required value of \( \bar{M} \) was derived from a sedimentation velocity experiment at a low solution density (\( \rho = 1.00473 \) g per ml) to avoid the use of high salt concentrations. The experimental details are given in Fig. 3 and the measured sedimentation coefficient was corrected for viscosity, density, and concentration dependence (\( k = 0.0089 \) ml per g (20)) to give \( s_{20, w} = 8.28 \) S. The use of the Svedberg equation with the value of \( D_{20, w} = 1.90 \times 10^{-7} \) cm² per s measured for various LDL samples (21) gave an apparent \( \bar{M} = 2.74 \times 10^4 \), in reasonable agreement with the values previously determined for LDL samples including that found in a sedimentation equilibrium experiment conducted at low density (18) where the results in Table I would suggest that the actual behavior is difficult to distinguish from that of a single solute.

The value \( \bar{M} = 2.74 \times 10^4 \) may now be used in conjunction with the results of the sedimentation equilibrium experiment at \( \rho = 1.03212 \) g per ml (Fig. 4A) to determine the corresponding value of \( \bar{v} \). The standard deviation, \( \sigma_2 \), of the partial molar volume distribution is available from that of the \( g(t_i) \) distribution, \( \sigma_2 \), using Equation 18c. This permits \( c(r) \) versus \( r \) to be simulated for the required experimental conditions via Equations 13, 10, 14, 15, 3b, and 4a, for assumed values of \( \bar{v} \) until satisfactory agreement with the experimental curve is obtained. The result is shown as the solid line in Fig. 4A and the partial specific volume, \( \bar{v} \), corresponding to \( \bar{M} \) was found in this way to have the value 0.9671 ml per g, in good agreement with the measurement 0.965 ± 0.004 ml per g when account is taken of the considerations discussed above under point 2.

The values of \( \bar{M} \) and \( \bar{v} \) may then be used in Equations 17a, 18a, 10, 12, and 24 together with the \( g(t_i) \) distribution to transform distributions in partial molar volume, molecular weight, and density of the LDL sample which are presented in Fig. 5, B, C, and D. The former distributions suffice to characterize the LDL sample in terms of the means and standard deviations reported in the...
Fig. 5. Experimentally determined distributions describing the human serum LDL sample. A, solid points show the experimentally determined distribution of \( t \), based on an analysis of the results shown in Fig. 4A and Equation 28 with \( \bar{e}(r_m) = 5.287 \) g per liter. The solid line through these points is a Gaussian curve computed with \( t = 0.09 \) and \( \sigma_t = -0.42 \). B, Gaussian distribution of \( M_t \) derived from Fig. 5A and characterized by \( \bar{M} = 2.74 \times 10^6 \) and \( \sigma_M = 0.29 \times 10^6 \). C, corresponding Gaussian distribution of partial molar volume with \( \bar{V} = 2.64 \times 10^6 \) and \( \sigma_V = 0.30 \times 10^6 \). D, non-Gaussian distribution of density, \( \rho_i = 1/\bar{\rho}_i \), obtained from Fig. 5A using Equation 24.

The caption of Fig. 5 for the Gaussian distributions; whereas the latter is presented because density is the parameter usually employed to characterize LDL samples. It is noted that the range of values of \( \rho_i \) shown in Fig. 5D is entirely consistent with the fractionation limits used to prepare the sample. It is also evident that this distribution is non-Gaussian, a property which may not be revealed in a histogram obtained using a limited number of equal density increments in fractionation studies.

In summary, the basic distribution which emerges from an analysis of a plot of \( c(r) \) versus \( r \) obtained in a sedimentation equilibrium experiment is a plot of \( g(t_i) \) versus \( t_i \). It could be noted that the \( g(t_i) \) values so obtained refer to the relative amounts of species in the cell, which equal the corresponding amounts in the original solution only if chemical interactions between the species are absent (23, 24). In general, such interactions may be detected in preliminary studies of the concentration dependence of a weight average property of the system such as a weighted sedimentation coefficient (25).

There is strong evidence of this type (21, 26) that the complication of chemical interaction does not arise in the study of LDL samples at the pH and ionic strength values used in this work (26). It could also be noted that Equations 8, 16, 19, and 22 (or its equivalent Equation 26) on which the evaluation of a plot of \( g(t_i) \) versus \( t_i \) is based make no assumption as to the form of this distribution and indeed Donnelly (11) has generated a non-Gaussian distribution of molecular weight (constant partial specific volume) with their use. It could be argued that the results found with the present LDL sample also indicate slight deviation from Gaussian form in \( t_i \); for indeed the sedimentation velocity peak (Fig. 3) exhibits slight asymmetry on the leading edge and the solid line in Fig. 5A does not exactly fit the experimental points. However, in view of experimental error and the empirical nature of Equation 26 used to fit the sedimentation equilibrium distribution, it was felt that an emphasis on the slight apparent skewness in Fig. 5A was unwarranted. It would have been possible, for example, to transform each \( g(t_i), t_i \) point in Fig. 5A to the corresponding distributions (Fig. 5, B, C, and D) without the use of the Gaussian transforms (Equations 17a, 18a, 10, 12, and 24) and \( \sigma_t = -0.42 \) provided that a suitable reference point \( (\bar{M}, \bar{V}) \) could be assigned. However, it is clear from Fig. 4A that the use of this \( \sigma_t \) does provide a very reasonable description of the basic experimental results within experimental error. Moreover, the comparison is sensitive to the value of \( \sigma_t \). Thus, a plot of \( c(r) \) versus \( r \) computed with \( \sigma_t \) increased from its estimated value of \(-0.42 \) to \(-0.49 \), with \( \bar{M} \) and \( \bar{V} \) as before, fell outside the error range indicated in Fig. 4A. This permits specification of the following maximum error limits on the standard deviations reported in Fig. 5: \( \sigma_t = -0.42 \pm 0.07 \), \( \sigma_M = 0.29 \pm 0.05 \times 10^6 \), and \( \sigma_V = 0.30 \pm 0.05 \times 10^6 \).

In conclusion, it is hoped that the theory presented, the methods of curve-fitting described which obviates the necessity of differentiating the experimental sedimentation equilibrium distribution, and the illustration of their use in characterizing a LDL sample may prove useful in the study of other systems of similar kind; for, indeed, the method is one which is economical in time and material. It may, however, be necessary to employ the density gradient analysis procedure (4) when systems of different kind are encountered, such as those exhibiting distinct bimodality of \( t_i \) in sedimentation velocity analysis; for, in these cases, such bimodality may be obscured (or be reflected only as asymmetry) in the \( g(t_i) \) versus \( t_i \) plot obtained by fitting a smooth plot of \( c(r) \) versus \( r \) to a function of the form given in Equations 20 or 28 (12).

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