CHANGES IN THE ELECTRIC DIPOLE VECTOR OF HUMAN SERUM ALBUMIN DUE TO COMPLEXING WITH FATTY ACIDS

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ABSTRACT The magnitude of the electric dipole vector of human serum albumin, as measured by the dielectric increment of the isoionic solution, is found to be a sensitive, monotonic indicator of the number of moles (up to at least 5) of long chain fatty acid complexed. The sensitivity is about three times as great as it is in bovine albumin. New methods of analysis of the frequency dispersion of the dielectric constant were developed to ascertain if molecular shape changes also accompany the complexing with fatty acid. Direct two-component rotary diffusion constant analysis is found to be too strongly affected by cross modulation between small systematic errors and physically significant data components to be a reliable measure of structural modification. Multicomponent relaxation profiles are more useful as recognition patterns for structural comparisons, but the equations involved are ill-conditioned and solutions based on standard least-squares regression contain mathematical artifacts which mask the physically significant spectrum. By constraining the solution to non-negative coefficients, the magnitude of the artifacts is reduced to well below the magnitudes of the spectral components. Profiles calculated in this way show no evidence of significant dipole direction or molecular shape change as the albumin is complexed with 1 mol of fatty acid. In these experiments albumin was defatted by incubation with adipose tissue at physiological pH, which avoids passing the protein through the pH of the N-F transition usually required in defatting. Addition of fatty acid from solution in small amounts of ethanol appears to form a complex indistinguishable from the "native" complex.

INTRODUCTION

Albumin-Fatty Acid Complexes

The complex formed by the binding of long chain fatty acids to serum albumin is in a number of respects unique among the great variety of complexes formed between small molecules and blood proteins. While at least 26 and perhaps as many as 70 mol of fatty acid can complex with albumin (Goodman, 1958; Spector et al., 1969), the unique characteristics are involved in the binding properties of the first 2 or 3 mol which attach at the so-called "high affinity" sites from which most other ligands (with the notable exception of bilirubin [Odell, 1973]) are excluded. Competitive binding

with other ligands occurs generally only with the third and subsequent moles of fatty acid bound, although allosteric effects occur at lower levels (Spector et al., 1973).

Fatty acid bound at the high affinity sites appears to play a role in stabilizing the albumin (Sogami et al., 1969; Boyer et al., 1946). Further, it appears under normal conditions to be unavailable to some tissue pools of free fatty acid (Higgins and Green, 1967). In vitro dissociation of the tightly bound fatty acid is particularly difficult to achieve, requiring special techniques (Goodman, 1957; Chen, 1967; Scheider and Fuller, 1970) which in most cases involve exposure to pH levels below the N-F transition.

These facts taken together suggest that the high affinity sites for fatty acid are interior to the normally accessible protein surface, a picture which is readily accomodated by the tertiary structure of albumin proposed by Brown et al. (1971, 1974); in this view, the acid-induced liberation of the fatty acid results from an opening up of the tertiary protein structure.

Dipole Measurements

The magnitude of the dipole vector and the time constants of rotary diffusion have long been used as measurable parameters to obtain certain kinds of structural information about protein molecules in solution (Oncley, 1943). While absolute structural information obtainable in this way is quite limited, these parameters are in many instances sensitive indicators of *changes* occurring in the molecule. The parameters reflect changes in charge configuration due either to ion binding or to charge rearrangement resulting from a change in the molecular conformation. To a lesser extent the molecular conformation itself is reflected in the time constants.

In this study we measured changes in the dielectric properties of human mercaptalbumin resulting from its complexing with long chain fatty acids. Similar measurements were also conducted on bovine mercaptalbumin with results generally consistent with data reported recently by Soetewey et al. (1972).

In addition to the obvious clinical interest in the human protein, two other factors give particular importance to the measurements on human albumin:

(*1*) The magnitude of the dielectric increment, and especially the magnitude of its dependence on the level of fatty acid binding, are both almost three times as great in human albumin as in bovine; and,

(*2*) In the case of human albumin, the change in dipole vector is the best and perhaps the only rapidly measurable indicator available for use in kinetic studies of the binding reaction. When fatty acid is bound to *bovine* albumin, there is a measurable change in that protein's tryptophan fluorescence intensity (Spector and John, 1968), but the particular tryptophan residue responsible for this change is absent in human albumin, and there is consequently no sufficiently sensitive rapid optical indicator for the complexing reaction.

With present technology, a dipole magnitude measurement can be completed and recorded in considerably shorter time than the 100 μs resolution time of stop flow kinetic apparatus.

MATERIALS AND METHODS

Our human mercaptalbumin (HMA) was prepared by Dr. S. Allerton, according to the method of Hughes and Dintzis (1964). Bovine mercaptalbumin (BMA) was prepared by Dr. T. Thompson using the same method. In addition we conducted a measurement on a sample of bovine serum albumin (BSA) which for undeterminable reasons was fat free in the condition in which it was supplied by Mann Research Laboratories of New York, identified as Lot F3815.

Before dielectric measurements were performed, the albumin solutions were deionized in an ion-exchange column in which all small ions were first exchanged for acetate and ammonium ions to avoid localized pH extremes in the subsequent passage over mixed bed H^+ and OH^- ion exchangers.

The fatty acid content of the protein solutions was assayed by the method of Dole (1956). Oleic acid was complexed to defatted or native albumin by addition in small amounts of ethanol by a method described by Soetewey et al. (1972). Protein concentration was determined by the method of dry weights.

Defatting at Physiological pH

The two relatively mild methods known for removing bound fatty acid from albumin preparations (Chen, 1967; Scheider and Fuller, 1970) both involve incubating the protein at a pH below the N-F transition. While much evidence suggests that the protein is undamaged by such an incubation, the defatting process used in this study, though somewhat less effective, avoided exposure to this pH range, giving added assurance that the native state of the protein had been preserved as well as is possible.

The method used takes advantage of the observation that the epididimal fat pads of the rat compete strongly, under certain conditions, for even the most tightly bound fatty acids on albumin (Bally et al., 1960).

In our process, a 50 mg/ml albumin solution is made in Krebs-Ringer phosphate buffer containing 4 mg/ml glucose and 0.04 μg/ml crystalline beef insulin, and is adjusted with 0.01 N KOH to pH 7.4 (Gordon and Cherkes, 1958). Into 5 ml of this solution, one drops the freshly dissected epididimal fat pads, briefly rinsed, from a 6-wk old 200 g white rat. The dissection is done with moderate precautions for sterility. The mixture is incubated in a shaker at 37° for 6 h. At 3 h and again at the end, the fat pads are removed and the solution is frozen and thawed for suppression of bacterial growth.

Small molecules are dialized out of the defatted solution. Protein contamination from all sources (insulin, and blood and related protein from the pads) is found to total less than one part in 50 parts of albumin.

By this method the fatty acid content of a "native" preparation, HMA I, was reduced from 1.1 mol/mol to 0.22 $\pm$ 0.05 mol/mol. The defatted albumin is identified as HMA II.

Dielectric Measurements

Dielectric measurements were made in a cell bounded by concentric cylindrical electrodes of 24K electrodeposited gold, having a sample volume of 4 ml and a cell con-

stant of 5.92 cm. A grounded guard ring was employed to reduce electric field fringe effects.

Impedance measurements were made using a custom-built substitution bridge built by Hollies and Oncley (1950) in which resistance elements were individually compensated to minimize reactive components. Measurements were made at 40 approximately logarithmically spaced frequency intervals between 1 kHz and 8 MHz. Sample temperature was (0.3 ± 0.3)° except in those data which were taken for analysis of the dispersion for relaxation times, for which the sample temperature was controlled to ±0.01°. The low sample temperature was chosen to reduce solution conductance and to maximize the relaxation times, bringing the dispersion range to frequencies at which more accurate measurements are possible.

ANALYSIS OF DIELECTRIC DATA

Based on the theory which relates dielectric measurements to molecular parameters of proteins in solution (Oncley, 1943; Debye, 1929; Scheider, 1965, 1970; Hendrickx et al., 1968) two distinct kinds of information are available: total increment and frequency dispersion.

Total Increment

From the *total dielectric increment*, $\Delta\epsilon$, defined as the difference between the low and high frequency asymptotic values of the dielectric constant, one obtains a value for the mean square molecular dipole vector magnitude, $\overline{\mu^2}$, which provides a measure of the electrical asymmetry of the charge distribution on the molecule. In any consistent units, $\Delta\epsilon$, a real quantity, is

$$\Delta\epsilon = \epsilon(\omega_0) - \epsilon(\omega_\infty) = (h/3)(n/3kT)\overline{\mu^2}, \tag{1}$$

where n is the number of molecules per unit volume, kT is the thermal energy term, and $(h/3)$ is a "reaction field" factor which can be estimated empirically (Oncley, 1943) to have the value 1.9 based on the measured dielectric increment of glycine, $D_{sp}(\text{gly}) = 0.35/\text{g/liter}$ (see Eq. 2), and a theoretical dipole moment of glycine $\mu(\text{gly}) = 15.0$ D (debye units: 1 D = 3.33×10^{-28} C-cm).

In conventional units, the total increment is expressed as a "specific dielectric increment," D_{sp}, in dielectric units normalized at the dielectric constant of free space, ϵ_0, and expressed per unit protein concentration in grams per liter of solution,

$$D_{sp} = \frac{\Delta\epsilon/\epsilon_0}{\text{protein conc. } (g/l)} = \frac{hN\overline{\mu^2}}{9000\,kT}\,\frac{1}{\epsilon_0}\,\frac{1}{\text{MW}}, \tag{2}$$

where N is Avogadro's number, MW is the molecular weight of the protein, taken as 66,000 for albumin, and $\epsilon_0 = 8.85 \times 10^{-14}$F/cm = $1/4\pi$ esu/statvolt-cm.

The total specific increment is thus a single numerical quantity for each sample. It can be determined with good accuracy, and one molecular parameter, the mean square dipole moment, can be derived from it directly. When this quantity changes signifi-

cantly as the result of a chemical modification of the protein molecule, it provides a good index of the state of the molecule with respect to that modification.

Frequency Dispersion

The second type of information is contained in the *frequency dispersion* of the dielectric constant, and is related to the molecular size and shape and to the direction of the mean dipole axis.

This information is contained not in a single measurable quantity, but in the variation of the complex dielectric constant, $\epsilon^*(\omega)$, over a frequency range of two to three decades. The derivation of molecular parameters from these data is complicated by the fact that often a number of different sets of values of these parameters fit the data nearly equally well, and the correct values may in fact not be those which represent the closest fit. Stated another way, the relevant information from the dispersion characteristics is subject to severe distortion from small artifacts, either experimental or mathematical. The manner of analysis of the data is thus a matter of great importance.

Considerable attention was therefore devoted in this study to techniques of analysis of dielectric dispersion data, including a critical examination of the reliability of the commonly used two-component representation, and the development of a more informative method.

Reliability of the Two-Relaxation Time Representation. In the most general terms, according to the classical Debye theory (1929), the dielectric increment referred to its asymptotic value, $\epsilon(\omega_\infty)$, at the high frequency end of the molecular rotation dispersion region, can be written as the superposition of a continuous spectrum $B(\tau)$ of components with relaxation time, τ, as follows,

$$\epsilon^*(\omega) - \epsilon(\omega_\infty) = \int_{1/\omega_\infty}^{\infty} B(\tau)/(1 + i\omega\tau)\mathrm{d}\tau, \tag{3}$$

where $i = (-1)^{1/2}$, ω is the imposed angular frequency of the measurement, and the asterisk denotes a complex quantity.

The form, Eq. 3, cannot in general be inverted in closed form, and so for computation $B(\tau)$ is approximated by a discrete spectrum S_N of N coefficients, $b(\tau_n)$, $n = 1, \ldots, N$, and this spectrum is obtained by solving the set of simultaneous equations, one for each of M data points,

$$\epsilon^*(\omega_m) - \epsilon(\omega_\infty) \approx \sum_{n=1}^{N} [b(\tau_n)/(1 + i\omega_m\tau_n)] \qquad m = 1, \ldots M. \tag{4}$$

Such a set of equations is easily solved in a least-squares sense if one seeks a representation in terms of only the best two-component spectrum $S_2(N = 2)$.

Perrin (1936) showed that the rotary Brownian movement of a particle whose shape is an ellipsoid of revolution can be characterized by two rotary diffusion constants, Θ_a and Θ_b, corresponding to rotation about the a and b axes, respectively, of the ellip-

soid, and that these properties are manifested in two observable relaxation times, τ_1 and τ_2. In addition, Edsall (1953) pointed out that only a prolate ellipsoid manifests two relaxation times differing by more than 10%, ruling out the oblate form from consideration as a model for the albumin molecule.

Thus, while the attempt to model the shape of a rigid molecule such as albumin in terms of an ellipsoid leads naturally to an attempt to resolve the dielectric dispersion data into the two components which produce the best fit, the question must be considered: Are the two relaxation times thus obtained likely in fact to correspond to the relaxation times of the molecule (even assuming that the ellipsoidal representation of the molecule [Oncley, 1942] is appropriate)? The assumption is usually made that they do. We proceed to examine this assumption, and will show that very small uncertainties in measurement can result in large uncertainties in the relaxation times calculated.

It is evident that any components of the system under measurement which are not orthogonal in a mathematical sense to the two components of S_2 will in some way be reflected in the two-component spectrum. We find, in fact, that such a spectrum is extremely sensitive to small variations in the manner of estimating the low and high frequency asymptotic behavior of the dispersion curve, and also to components of small magnitude in a dispersion region well away from the frequency range where molecular relaxation is assumed to be involved. This is especially true when, for mathematical simplicity, the problem is reduced to a three-parameter system by normalizing the sum of the coefficients, in effect weighting the least reliable data most heavily.

In practice, the low frequency asymptote is always subject to small errors in correcting for electrode polarization and often to small additional components due perhaps to relaxation of molecular aggregates. Estimates of the high frequency asymptote are subject to small errors due to the difficulty in correcting for reactive circuit residuals at higher frequencies.

One indication that components other than the molecular rotation time constants are entering into the determination of S_2 components is the fact that the residuals have significant systematic component, suggesting that the representation contains too few degrees of freedom.

The extreme sensitivity of S_2 to small nonmolecular components is illustrated by simulated data in Fig. 1. Here perfect data from a two-component system have been artificially perturbed by addition of a 5% component at a low frequency and by a misestimation of the high frequency asymptote by an amount equal to 2% of the increment. The latter error would result from a misestimation by only 0.04 pF in a 0.5% solution of human albumin in a cell of 6 cm cell constant, or a misestimation by only 0.015 pF in a similar solution of bovine albumin. These very small experimental errors result in a distortion of the S_2 spectrum to the extent of about 25% in both the time constants and the coefficients.

Our conclusion is that any molecular parameters derived from two-component fitting of dielectric dispersion data have uncertainties of the order of 25%.

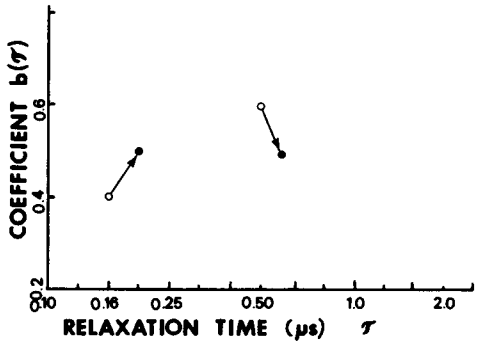

FIGURE 1

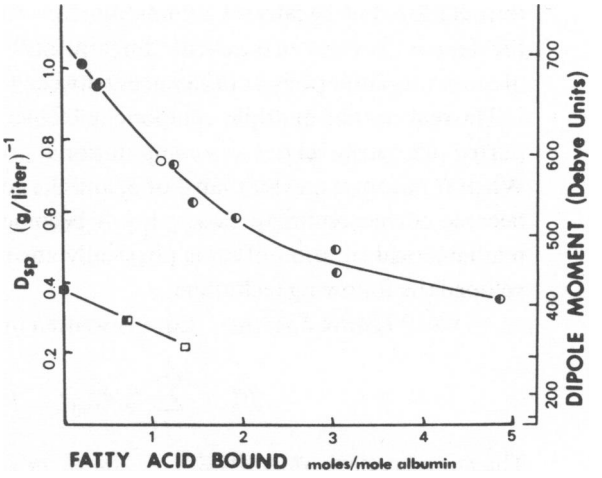

FIGURE 2

FIGURE 1 Mapping of least-squares two-component solutions for simulated data, showing the sensitivity of such solutions to small systematic errors. ○, stimulated data containing two components of magnitude 0.6 and 0.4 with relaxation times of 0.5 μs and 0.16 μs, respectively. ●, the same data with an additional component of magnitude 0.05 and relaxation time of 16 μs and with a high-frequency asymptote misestimation error of 2% of the total increment.

FIGURE 2 Total dielectric increment per gram/liter and calculated root mean square molecular dipole moment for albumin solutions of concentration 10 g/liter at 0.3°, as a function of moles of fatty acid bound. ○, native human mercaptalbumin (HMA I); ●, defatted human mercaptalbumin (HMA II); ◐, defatted human mercaptalbumin with oleic acid added back in vitro; □, native bovine mercaptalbumin; ■, bovine serum albumin, fat free, as obtained from commercial supplier; ◧, fat-free BSA with oleic acid added in vitro. (Data from Dintzis, 1952, and Scheider, 1962*b*.)

Multiple Relaxation-Time Spectra and Modified Two-Component Representation. We have found that these difficulties are significantly diminished in a multiple relaxation-time analysis. The reason for this is principally that components outside the region of molecular relaxation are expressed in separate components, and thus the principal portion of the spectrum more accurately reflects what is going on in that portion alone.

This, in fact, suggests the use of a multiple relaxation spectrum as a means of improving the accuracy of a two-component representation, if it is specifically desired to obtain an S_2 description because of the interest in describing the molecule in terms of the ellipsoidal model. The modified S_2 procedure involves subtracting from the data the peripheral components revealed by the multiple-component spectrum, and then obtaining a two-component spectrum. This procedure was followed in the analysis of HMA II data represented by the squares in Fig. 4, with the result that what had appeared to be a significant divergence of molecular characteristics between HMA I and HMA II could be accounted for entirely by peripheral components from an S_{12} analysis, components almost certainly not related to molecular rotation rates.

The multiple-component spectrum itself is less directly amenable to physical in-

terpretation, but because it retains more of the significant information contained in the data, it provides an excellent "fingerprint," or recognition pattern, for the purpose of assessing similarities or differences between two samples.

The reason that multiple component Debye spectra of this sort have not been reported previously relates to a computational problem which is primarily mathematical: When N becomes greater than 2 or 3, and the relaxation times of adjacent components become correspondingly closer, Eq. 4 becomes increasingly ill-conditioned, and the mathematical solution often is physically absurd. To overcome this problem, we developed the following technique.

Non-Negative Spectra. Eq. 4 is written in matrix form,

$$p_m^* \approx \sum_{n=1}^{N} b_n A_{m,n}^* \qquad m = 1, \ldots, M. \tag{5}$$

The complex data $p_m^* = \epsilon^*(\omega_m) - \epsilon(\omega_\infty)$, $m = 1, \ldots, M$, which may be weighted according to estimates of their precision, are to be fitted in a least-squares sense by N spectral components, $A_{m,n}^* = (1 + i\omega_m \tau_n)^{-1}$, whose relaxation times, τ_n, are fixed at the outset, usually at logarithmic intervals. The spectrum S_N consists of the N coefficients, b_n.

A conventional least-squares regression would be accomplished by inversion of the matrix product of $\mathbf{A}^*$ with its transpose. This method always gives the closest possible fit in the mathematical sense. In practice, if $\mathbf{A}^*$ is ill-conditioned, the solution which is in this sense the "best," frequently is a wildly oscillatory function containing large alternately positive and negative coefficients which completely mask the physically meaningful information. (Coefficients from a Debye system matrix, $N = 12$, $M = 40$, with relaxation times spaced 5 per decade, are typically several orders of magnitude larger than the "true" values to be expected.)

Similar difficulties were reported by Wiff and Gehatia (1973) in their efforts to interpret sedimentation equilibrium data in terms of a molecular weight distribution. They appropriately describe an ill-conditioned inversion matrix such as $(\mathbf{A}^{*\mathrm{T}}\mathbf{A}^*)^{-1}$ as an error amplifier.

Mathematically, this problem arises when the cone encompassing all physically realizable combinations of the N vectors $\mathbf{A}_n^*$ with components $A_{m,n}^*$ in M-space, is narrow such as to cause even small experimental error to displace the data vector $\mathbf{p}^*$ outside the cone.

This can be understood in principle as follows: Define adjacent difference vectors,

$$\boldsymbol{\delta}_{n,n+1}^* = \mathbf{A}_n^* - \mathbf{A}_{n+1}^*, \tag{6}$$

which will be nearly orthogonal to both $\mathbf{A}_n^*$ and $\mathbf{A}_{n+1}^*$, and which will not correspond to any physically realizable component. Nevertheless the $\boldsymbol{\delta}$ vectors are mathematically available to the solution of the set of equations 5, and their inclusion in such solutions results in the physically absurd spectra which often are obtained. In such instances the residuals are typically much smaller than the experimental uncertainty of the data, indicating that the mathematics has forced the spectrum to use physically

unrealizable combinations of components to minimize residuals beyond the point where these residuals contain any component of physical significance. Since the δ vectors are small in magnitude, they generate large coefficients.

In such solutions, the smallness of the residuals is deceptive, and does not indicate a valid fit; a proper index of the limiting factors involved in the validity of a fit has been previously discussed (Scheider, 1962*a*).

The way out of these difficulties was found by restricting the resultant represented by the spectrum to fall within the cone of physically realizable solutions. This involves restricting the spectrum to non-negative coefficients, $b_n \geq 0$. This required replacing the straightforward least-squares regression method by an iterative inversion of the matrix Eq. 5 in which estimates of the coefficients are successively improved by a modified relaxation method the details of which are presented elsewhere (Scheider, 1962*b*). The modification consists essentially of aborting any iteration which would cause a coefficient to become negative, and substituting for such an iteration the next best permitted iteration.

By this method, we have obtained a number of very believable and self-consistent spectra, including those in Fig. 3 for HMA I and HMA II at various concentrations.

It is to be emphasized, however, that the method derives its justification not from the fact that it "works," but rather, from the fact that it is consistent with, and is in fact *required by,* the Least Squares Theorem, stated in full rigor (Plummer, 1940), as follows: "Of all solutions *with equal a priori likelihood,* that one is most probable which minimizes the sum of the squares of the residuals." This requires that all of the a priori evident physical constraints upon the solution be applied before the best fit in the least squares sense is chosen. An obvious constraint is that any spectrum containing a negative coefficient has an a priori probability of zero.

Wiff and Gehatia (1973) additionally applied a "regularization" to the treatment of their problem, by which the distribution function was smoothed through imposition of further adjustable criteria minimizing not only residuals but also the derivatives of the distribution function. We find in the present case no physical justification for imposing such criteria.

ASYMPTOTE ESTIMATION

In these experiments, the high frequency asymptote, $\epsilon(\omega_\infty)$, was estimated by extrapolation of the data from the points between 2 and 8 MHz on the assumption that for this purpose a single Debye component adequately approximates this tail of the data.

The low frequency asymptote, $\epsilon(\omega_0)$, is in general masked by the effect of electrode polarization capacitance. This is, in effect, a capacitive component interposed in series between the sample and the electrodes; its magnitude was estimated by an empirical method based on a theory of the frequency dispersion of polarization (Scheider, 1975), and consists in determining for each run the parameters, α and C°_{pol}, of the expression,

$$C_{pol}(\omega) = C^\circ_{pol}\omega^{-\alpha}. \tag{7}$$

These parameters were estimated from impedance measurements made for this purpose at very low frequencies (250 Hz to 1 kHz), where the frequency dependence due to polarization dominates; α was found to be between 0.2 and 0.3 in these experiments.

RESULTS

The most pronounced changes resulting from addition of fatty acid to albumin are in the total dielectric increment. Values of total increment for the samples of HMA, BMA, and BSA as a function of bound fatty acid are shown in Fig. 2. These data are from solutions of low protein concentration (approximately 10 g/liter). The concentration dependence of total increment introduces an uncertainty of less than 3% in these values; such dependence is more pronounced in human albumin than in bovine.

The results for bovine albumin are probably consistent with values reported by Soetewey et al. (1972); it must be assumed that the data in the cited reference were obtained at room temperature.

Spectral analysis of the dispersion curves was performed on the two samples of human mercaptalbumin, HMA I, containing 1.1 mol of physiologically bound fatty acid, and HMA II, defatted to a level of 0.22 mol by the method described. The S_{12} spectral profiles are shown in Fig. 3. Included are profiles obtained from sets of data representing the extrapolation to zero protein concentration from the series for each sample.

It should be emphasized that the present method has greatly reduced, but not eliminated, the mathematical artifacts introduced by the ill-conditioned nature of the system of equations as discussed above. The resulting spectra thus, (*a*) fit the data exceedingly well, (*b*) are excellent representations of the mathematical properties of the data, (*c*) indicate rather well the location on the logarithmic time scale where the principal physical relaxation of the sample occurs, but (*d*) are not to be used for assigning physical significance to the precise magnitude of any single component. Spectra obtained from the data extrapolated to zero concentration are subject to the additional exaggeration of all errors inherent in extrapolation; thus, no physical significance can be assigned, for example, to the unexpected appearance of the component at $\tau = 8\ \mu s$ in the extrapolated HMA II spectrum.

The spectra show, as expected, a broadening and a movement toward longer relaxation times, with increasing protein concentration, due in part at least to the increased viscosity and the greater heterogeneity of the immediate environment of the protein molecules. Moser et al. (1966) reported for the principal relaxation time observed in electric birefringence decay a similar concentration dependence; however, Wright and Thompson's (1975) two-component spectra showing the slow component to be virtually concentration-independent would seem to be inconsistent with the present results.

Two-component spectra, S_2, for the same samples of HMA I and HMA II, are shown in Fig. 4. Without the modification described above, these spectra would suggest that removal of the fatty acid has resulted in a swelling of the molecule (longer relaxation times), and a shift in the direction of the dipole vector toward the short

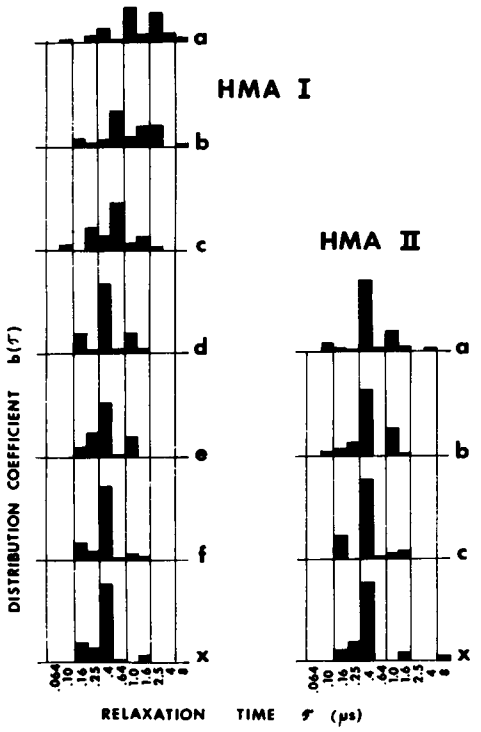

FIGURE 3

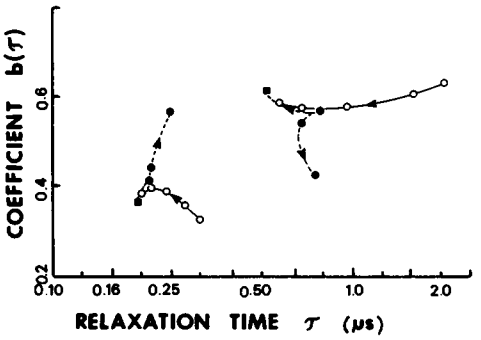

FIGURE 4

FIGURE 3 Relaxation spectrum profiles for human mercaptalbumin. HMA I, native protein, (*a*) 93.5 g/liter, (*b*) 71.7 g/liter, (*c*) 35.2 g/liter, (*d*) 13.8 g/liter, (*e*) 9.3 g/liter, (*f*) 5.3 g/liter, (*x*) data extrapolated to zero concentration; HMA II, defatted protein, (*a*) 13.4 g/liter, (*b*) 7.5 g/liter, (*c*) 4.1 g/liter, (*x*) extrapolated to zero concentration. The concentration series indicates the effect of retarded rotation due to high concentration in the more diffuse profile, shifted in the direction of longer relaxation times. At low concentrations, the components with relaxation times of 1 μs or longer are believed to reflect low frequency asymptote errors (see text).

FIGURE 4 Mapping of least-squares two-component solutions for dielectric dispersion of human mercaptalbumin. ○, native HMA I, at five concentrations, 93.5, 71.7, 35.2, 13.8, and 5.3 g/liter; arrow points in the direction of diminishing concentration. ●, defatted albumin, HMA II, at 13.4, 7.5, and 4.1 g/liter. ■, HMA II at 4.1 g/liter in which the solution was obtained by first subtracting from the data a long relaxation time component of magnitude 0.085 and relaxation time 1.6 μs, as revealed by spectral analysis (Fig. 3); the result indicates that the apparent divergence between the two series of solutions obtained from the raw data may not be real.

axes (changes in the magnitude of the coefficients). This conclusion, however, is not substantiated by comparison of the S_{12} spectral profiles of HMA I and HMA II.

The two-component analysis of the 4.1 g/liter sample of HMA II, when modified according to the method described above, results in the disappearance of the apparent molecular changes suggested by the unmodified two-component analysis. This conclusion is consistent with the S_{12} profiles.

Even though the modified S_2 method has brought the results into consistency with the more reliable S_{12} representation, our conclusions regarding the large uncertainty (of the order of 25%) in the molecular parameters derived from any S_2 method remain.

Several points should be emphasized in regard to this assignment of a seemingly large uncertainty to such results: (*1*) a 25% uncertainty limit is equivalent to a $\frac{1}{10}$ decade uncertainty, which is not surprising in view of the fact that the parameters derive from relaxation measurements with intrinsically broad resonance peaks spanning in excess of a decade on the frequency scale; (*2*) The present error analysis can serve in part to provide an interpretation of varying recently reported data on albumin hydrodynamic properties (see Fig. 5) which in fact do span the range of uncertainty we have estimated, even though these results are often given with two or three significant figures without qualification, thus leading to over-interpretation, particularly with respect to shape differences; (*3*) Placing an outer limit of uncertainty does not preclude that the data may in fact be considerably more accurate, in particular where information from various techniques is used in combination to advantage (Moser et al., 1966; Squire et al., 1968). In combined data analysis other accuracy criteria come into play, principally estimates of the true independence of information and the possibility of common or reinforcing systematic error; these matters are beyond the scope of this discussion.

In terms of an ellipsoidal model of the HMA molecule, we would thus assert only that the rotational diffusion constants in water at 0.3° are $(1.00 \pm 0.25) \times 10^6\ s^{-1}$ and $(4.26 \pm 1.0) \times 10^6\ s^{-1}$; and that these values are equally applicable to the defatted albumin and to albumin containing one mole of fatty acid. Because of the general tendency of the two diffusion constants to co-vary under the types of error considered in the analysis, it is possible to restrict the uncertainty in the axial ratio a/b; calculated according to the equations of Perrin (1936), $a/b = 3.7 \pm 0.6$. We may also restrict the locus of possible combinations of axial dimensions a and b to the shaded area in the a,b plane of Fig. 5.

In Fig. 5, calculated values of the semiaxes a and b of ellipsoidal representations are given for these data and for other published results on serum albumins, which are

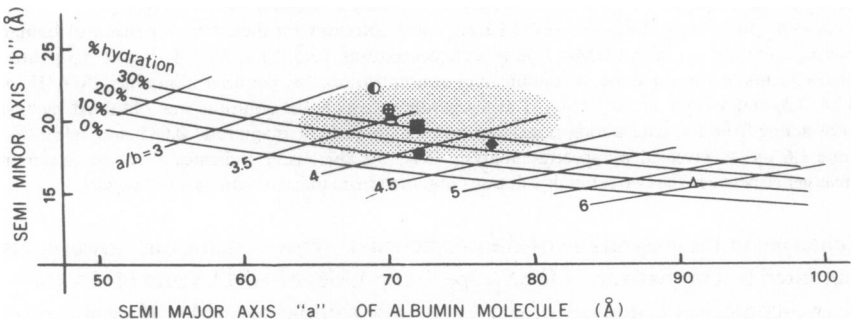

FIGURE 5 Calculated axial dimensions of ellipsoidal representation of serum albumin molecules from the data of Table I, plotted in the a,b plane, showing lines of constant molecular volume of albumin with different amounts of hydration. △, horse serum albumin, Oncley, 1942; ●, BSA, Moser et al., 1966, from dielectric data, and ⊕, same, except combined data from dielectric and birefringence decay methods; ⊖, BSA, Squire et al., 1968; ◆, BSA, Soetewey et al., 1972; ⊛, BSA, Wright and Thompson, 1975; ■, human serum mercaptalbumin, this work. The shaded area represents the estimated uncertainty around the central result of this work.

TABLE I

RESULTS OF TWO-COMPONENT RELAXATION TIME ANALYSIS OF DIELECTRIC DISPERSION (D) AND ELECTRIC BIREFRINGENCE DECAY (EB) OF SOLUTIONS OF SERUM ALBUMIN

Molecule	Experimental time constants reported		Temp.	Rotary diffusion constants calculated and corrected to 0°C ($\times 10^6$ s^{-1})		Semi-axes of ellipsoid of revolution ($\times 10^{-8}$ cm)		Axial ratio a/b	Method	Reference
	τ_1	τ_2		Θ_b	Θ_a	a	b			
	μs	μs	°C							
Horse serum albumin	0.36	0.075	25	0.66	5.6	91	15.4	5.9	D	Oncley, 1942
Bovine serum albumin, native and defatted	0.264	0.088	25(?)	0.89	4.5	77	18.3	4.2	D	Soetewey et al., 1972
	0.22	0.074	25	1.07	5.30	72	17.6	4.1	D	Moser et al., 1966
	0.077§	—	25	1.02	—	—	—	—	EB	"
	0.23	0.11	25	1.00	3.35	69	22.3	3.1	D/EB*	"
	—	—		—	—	70	20	3.5	D/EB‡	Squire et al., 1968
	0.078§	0.028§	25	1.02	3.73	70	20.7	3.4	EB	Wright and Thompson, 1975
Human serum mercaptalbumin native and defatted	0.50 +0.15/−0.10	0.19 +0.07/−0.04	0.3	1.00 ± 0.25	4.26 ± 1.0	72 ± 10	19.5 +3/−2	3.7 ± 0.6	D	This work

*Results estimated (by Moser et al.) from combined data of dielectric and birefringence methods.

‡This is a new analysis of the data of Moser et al. (1966) in conjunction with sedimentation equilibrium data.

§The experimental time constants from electric birefringence decay are not directly comparable with those obtained from dielectric relaxation; however the rotary diffusion constants derived from them are comparable with those calculated from dielectric relaxation time constants.

tabulated in Table I. The figure shows these points in relation to lines of constant molecular volume calculated from values of partial specific volume given by Hunter (1966) and adjusted to take into account various amounts of bound water. Our central values are seen to be consistent with the model of the molecule as an ellipsoid containing 30% water of hydration.

CONCLUSIONS

The large specific dielectric increment of human serum albumin (about three times that of the bovine protein), as well as the proportionately greater dependence of the increment on the binding of fatty acid, marks this molecule as a suitable protein in which to study this effect, and in turn makes the dielectric increment the most sensitive, if not the only, suitable indicator of the extent of the complexing reaction for kinetic studies.

The data suggest that the in vitro complexing as done in these experiments by addition in ethanol results in a complex which is structurally indistinguishable in terms of molecular configuration from the native formed complex.

The relaxation spectra, shown in the profiles of Fig. 3, appear to suggest no evidence of a significant change in dipole angle, mean radius, or shape factor, between defatted human mercaptalbumin and that containing one mole of bound fatty acid. This does not rule out the possibility that transient configuration changes occur during complexing.

The results of two-component relaxation analyses are given, along with reasons for assigning uncertainties of the order of 25% to any such results taken alone.

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