and substituting the second form given into eq 43 gives:

$$v^*_{A\rightarrow P} = \frac{[k_2k_3/(k_2 + k_3)][E_t]}{1 + k_2/k_1[A] + k_3/k_4[P]} \quad (46)$$

The rate of P* → A exchange must equal the rate of A* → P exchange, since the system is at equilibrium.

*Exchange at Equilibrium in Sequential Mechanisms.* A similar approach can be applied to sequential mechanisms where all reactants are present and the system is at chemical equilibrium. Equation 44 still applies, except that with the same number of terms in the denominator as there are enzyme forms, the rate constants for one step (the conversion of the last enzyme form back into $E_i$) will not appear in this denominator.

## Discussion

As described above, the calculation of net rate constants by partition methods allows one to write down directly a large number of useful kinetic expressions and rate equations. Thus $V$ is $E_t$ times the reciprocal of sums of reciprocals of the net rate constants when all substrates are saturating, and $V/K$ is normally $E_t$ times the net rate constant for combination of enzyme and substrate, divided by substrate concentration, when substrate level is very low. The net rate constant for combination of labeled reactant with enzyme, multiplied by the concentration of that enzyme form, gives the rate of isotope exchange, either in the steady state or at equilibrium. The technique works equally well for situations in the steady state, at equilibrium, or in a single turnover, as with the isotope partition example. No doubt further applications will become apparent with time. In this laboratory this technique has become the method of choice for routine derivations where it is desired to determine quickly the result of expanding a mechanism by adding extra steps with extra rate constants associated with them, and an immense amount of time has been saved thereby. Hopefully this paper will serve to make the method equally available to others with an interest in enzyme kinetics.

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# A Study of Protein–Sodium Dodecyl Sulfate Complexes by Transient Electric Birefringence†

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ABSTRACT: The method of transient electric birefringence has been applied to study the conformation of protein–sodium dodecyl sulfate complexes. A model of a deformable prolate ellipsoid has been proposed for the protein–dodecyl sulfate complex. This model is compared to the models proposed by J. A. Reynolds and C. Tanford ((1970), *J. Biol. Chem. 245,* 5161) and K. Shirahama, K. Tsujii, and T. Takagi ((1974), *J. Biochem. 75,* 309). Differences between these latter two models are resolved by the model presented here. In addition, it has been demonstrated that protein molecular weights may be obtained from the slow relaxation time for transient electric birefringence of protein–dodecyl sulfate complexes.

The generally accepted basis for molecular weight determination of proteins by sodium dodecyl sulfate gel electrophoresis has been proposed by Reynolds and Tanford (1970), who studied the hydrodynamic behavior of several protein–sodium dodecyl sulfate complexes by means of intrinsic viscosity. Their data suggested that the complex is a rod-like particle, the length of which varies uniquely with the molecular weight of the protein moiety.

The simplest model suggested by their data is a prolate ellipsoid, with constant semi-minor axis, and a semi-major axis which is proportional to the molecular weight of the protein moiety. The average value of the semi-minor axis is 18.1 Å with a range of 3.4 Å for 12 protein–dodecyl sulfate complexes at the binding level of 1.4 g of dodecyl sulfate/g of protein. The semi-major axis varies linearly with molecular weight over the range studied: cytochrome *c* (11,700) 44.8 Å to bovine serum albumin (69,000) 230.5 Å.

Recently, Shirahama et al. (1974) have proposed an alternative model of protein–dodecyl sulfate complexes, which they call the "necklace model". In this model, the polymer chain is flexible and micelle-like clusters of dodecyl sulfate are scattered along the chain. The authors state that the necklace model is at variance with the ellipsoid model in the following points: (1) the former is flexible and free-

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draining with respect to the solvent, but the latter is rigid and nondraining; (2) a large amount of $\alpha$-helical structure in the polypeptide chain is a prerequisite for the latter, but not for the former.

The experimental results on which Shirahama et al. base their model are twofold. (1) Free-boundary electrophoresis of nine protein–dodecyl sulfate complexes, for which the molecular weights of the polypeptides ranged between 10,000 and 70,000, yielded electrophoretic mobilities independent of the molecular weights of the polypeptides. (2) Electrophoretic mobilities of three dodecyl sulfate–poly(vinyl pyrrolidone) (PVP)[1] complexes were independent of the molecular weights of the synthetic polymers. The average molecular weights of the three PVP polymers were 24,500, 40,000, and 700,000. The electrophoretic mobilities of the dodecyl sulfate–protein and dodecyl sulfate–PVP complexes were almost identical, which suggested to the authors that the complexes were free-draining coils with no compact conformation. Additionally, the authors state that since PVP has no tendency to form $\alpha$-helical or any other ordered structure, the formation of a rigid and ordered conformation of the polymer, as presumed by Reynolds and Tanford, is not a requisite for insensitivity of electrophoretic mobility to molecular weight.

The purpose of this paper is to present the results of a different hydrodynamic method applied to a series of protein–dodecyl sulfate complexes, and to compare their results with the two alternative models for protein–dodecyl sulfate complexes discussed above. The method we have used is transient electric birefringence. For proteins which can be represented as equivalent prolate ellipsoids, it has recently been demonstrated that the dimensions of the particle may be uniquely determined by this method (Wright et al., 1974; Wright and Thompson, 1974; Wright and Fish, 1975). For sufficiently large axial ratio it has been demonstrated that the term of the rotational diffusion tensor corresponding to rotation of the long axis about the short axis may be determined by this method even though the second distinct term may not be observed (Wright and Keefe, 1970). This term, however, is sufficient for the determination of the axial ratio of the ellipsoid. Additionally, if the model of Reynolds and Tanford is correct, then it should be possible to determine protein molecular weight by all hydrodynamic methods for which theory for the prolate ellipsoid has been derived.

## Experimental Section

*Materials.* Analytical grade $NaH_2PO_4 \cdot H_2O$, $Na_2HPO_4 \cdot 7H_2O$, glacial acetic acid, ammonium persulfate, and methanol were obtained from Fisher Scientific Company. Acrylamide, methylenebisacrylamide, *N,N,N′,N′*-tetramethylethylenediamine, and $\beta$-mercaptoethanol were obtained from Eastman. The Coomassie Brilliant Blue R250 was a Colab product. Sodium dodecyl sulfate was obtained from the Pierce Chemical Company. The sources of the proteins used are listed in Table I.

*Acrylamide Gel Electrophoresis.* The method of Weber and Osborn (1969), with a minor modification, was used for the preparation of protein samples, gel electrophoresis, staining and destaining of gels, and measurement of mobility of protein samples into the gels. The proteins (1 mg/ml) were dissolved in 0.01 M sodium phosphate buffer (pH 7.0) that contained 1% (w/v) sodium dodecyl sulfate and 1% (v/v) $\beta$-mercaptoethanol. The solutions were heated at 90° for 20 min instead of the 2-hr incubation at 37° as used by the previous investigators (Weber and Osborn, 1969).

Table I: Proteins Used in Sodium Dodecyl Sulfate Complexes.

| Sample No. | Protein | Mol Wt | Source |
|---|---|---|---|
| 1 | Cytochrome *c* | 11,700 | Sigma |
| 2 | Lysozyme | 14,300 | Boehringer Mannheim |
| 3 | $\beta$-Lactoglobulin | 18,400 | Pentex |
| 4 | Chymotrypsinogen A | 25,700 | Worthington |
| 5 | Ovalbumin | 43,000 | Pentex |
| 6 | Bovine serum albumin | 69,000 | Sigma |
| 7 | Human transferrin[b] | 72,000[a] | Gift of Dr. Philip Aisen, Albert Einstein College of Medicine, New York, N.Y. |

[a] Polypeptide molecular weight for this glycoprotein of molecular weight 77,000 (Mann et al., 1970) corrected for 6% carbohydrate (Leach and Fish, 1974). [b] Sample 7 was measured 3 weeks after completion of experiments with samples 1–6.

*Measurement of Birefringence Relaxation Times.* The protein was dissolved in deionized water to a concentration of 2% (w/v). The sample was then made 1% (v/v) $\beta$-mercaptoethanol and 3% (w/v) dodecyl sulfate. The pH was adjusted to 8 with small amounts of 1 *N* NaOH. The samples were then heated at 90° for 20 min prior to dialysis against 6 l. of 0.1% (w/v) dodecyl sulfate and 0.002% (w/v) sodium azide in a continuous dialyzer. To account for concentration changes, the sample volumes were measured after completion of dialysis. The final binding ratio of dodecyl sulfate/protein does not change when the reducing agent is removed (Reynolds and Tanford, 1970; Fish et al., 1970). The viscosity of the solvent (0.1% dodecyl sulfate and 0.002% sodium azide) was measured in a Cannon-Fenske viscometer (water flow time 240 sec) immersed in a water bath thermostated to 25 ± 0.005°. The value determined was 0.8872 cP.

The birefringence relaxation times were measured, as previously described (Wright et al., 1974; Wright and Thompson, 1974), with an apparatus constructed in the Biophysics Department at the Medical College of Virginia. The instrument response is less than 5 nsec.

## Results

The birefringence relaxation times reported in this study were determined graphically from photographs of the transient birefringence pulse displayed on an oscilloscope (Wright et al., 1974; Wright and Thompson, 1974). In most cases, the decay curves were typical of the sum of two exponential decay processes. The slow relaxation times were determined from the slopes of the straight line asymptotes at long times on semilog plots of the decay curves. The fast relaxation times were determined from the slopes of straight lines fit to the differences between the observed decay curve and the slow relaxation curve determined at long times.

The slow and fast relaxation times determined for the protein–dodecyl sulfate complexes used in this study are shown in Table II, as a function of concentration. Blanks in this table appear for those cases in which the relaxation curves were single exponentials. Since the relaxation times were dependent on protein concentration, the limiting values at zero concentration were determined. These values

[1] Abbreviation used is: PVP, poly(vinylpyrrolidone).

Table II: Concentration Dependence of Fast and Slow Transient Birefringence Relaxation Time.

| | Protein Sample Number[a-c] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 (2.00) | | 2 (2.00) | | 3 (1.76) | | 4 (1.78) | | 5 (1.95) | | 6 (1.74) | | 7 (1.00) | |
| Dilution | $\tau_s$ | $\tau_f$ | $\tau_s$ | $\tau_f$ | $\tau_s$ | $\tau_f$ | $\tau_s$ | $\tau_f$ | $\tau_s$ | $\tau_f$ | $\tau_s$ | $\tau_f$ | $\tau_s$ | $\tau_f$ |
| 1:0 | | | 35.5 | 10.9 | 47.7 | 13.6 | 84.6 | 30.0 | 126.0 | 27.6 | 215.5 | 49.1 | 208.0 | 46.0 |
| 1:1 | 55.9 | | 33.4 | 13.4 | 49.1 | | 100.9 | 30.0 | 144.6 | 59.7 | 231.9 | 53.2 | 204.0 | 13.0 |
| 1:3 | 30.0 | 19.1 | 36.9 | | 49.1 | | 114.6 | 31.4 | 164.8 | 52.1 | 251.0 | 54.6 | 196.0 | 26.0 |
| 1:7 | 36.8 | | 39.6 | 15.0 | 45.1 | | 114.6 | 27.3 | 161.5 | | 259.2 | 47.7 | | |
| 1:∞[d] | 23.5 | 19.1 | 37.0 | 15.8 | 48.4 | 13.6 | 121.5 | 29.2 | 175.0 | 64.0 | 262.0 | 53.1 | 195.0 | 22.0 |

[a] See Table I for protein identification. [b] Numbers in parentheses are the protein concentrations, after dialysis, in g/100 ml. [c] Relaxation times are expressed in nsec. [d] Values extrapolated to zero protein concentration.

Table III: Semi-Axial Lengths for Prolate Ellipsoid Models for Several Protein–Sodium Dodecyl Sulfate Complexes.

| | Transient Birefringence | | Viscosity[a] | |
|---|---|---|---|---|
| Protein | *b* (Å) | *a* (Å) | *b* (Å) | *a* (Å) |
| Cytochrome *c* | 24.7 | 36.3 | 17.2 | 44.8 |
| Lysozyme | 18.4 | 53.6 | 18.9 | 45.4 |
| β-Lactoglobulin | 15.6 | 63.4 | 17.8 | 65.8 |
| Chymotrypsinogen A | 19.3 | 88.3 | 18.2 | 87.4 |
| Ovalbumin | 28.0 | 92.97 | 17.6 | 156.9 |
| Bovine serum albumin | 22.6 | 116.7 | 18.4 | 230.5 |
| Human transferrin | 14.7 | 113 | | |

[a] Calculations based on viscosity data of Reynolds and Tanford (1970).

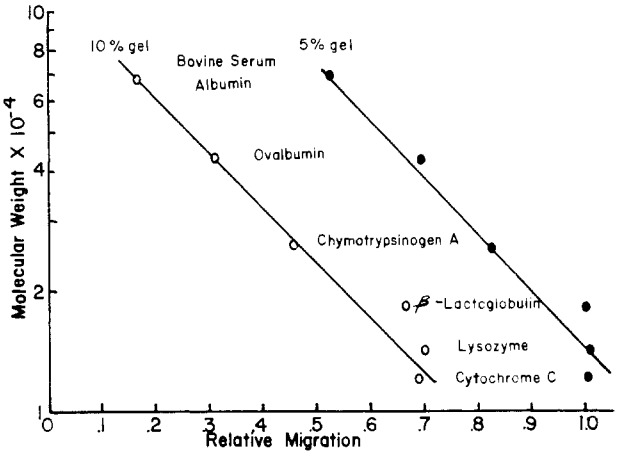


FIGURE 1: Molecular weight determined by sodium dodecyl sulfate gel electrophoresis.

are shown in Table II at infinite dilution. All calculations in this paper are based on the extrapolated relaxation times.

According to the general theory of Ridgeway (1966), prolate ellipsoids may be described by a double exponential decay, where the relaxation times are given as $\tau_s = 1/6R_1$ and $\tau_f = 1/2(R_1 + 2R_3)$. $R_1$ denotes the principle rotary diffusion coefficient for rotation of the semi-major axis about the semi-minor axis, and $R_3$ denotes the principle rotary diffusion coefficient for rotation about the semi-major axis. Therefore, these data are supportive of a prolate ellipsoid model for protein–dodecyl sulfate complexes, since two relaxation times were observed for each sample.

The rotary diffusion coefficients $R_1$ and $R_3$ were calculated from the fast and slow relaxation times of Table II, according to the above equations. These values yield estimates of *a* and *b*, the semi-major and -minor axes, respectively, according to the inversion procedure of Wright et al. (1973). Table III shows the values of *a* and *b* calculated from the transient birefringence data for the protein–dodecyl sulfate complexes used in this study. These dimensions are compared to those calculated by Reynolds and Tanford (1970) based on viscosity and binding data. The agreement between the two sets of dimensions is good for all proteins whose molecular weight is less than or equal to 25,700. The lack of agreement between the dimensions for bovine serum albumin and ovalbumin, calculated by the different methods, is not understood; however, such differences have been reported by other investigators (Bur and Fetters, 1973). It may reflect on the assumption that protein–dodecyl sulfate complexes are rigid prolate ellipsoids. A rigid prolate ellipsoid may only be an approximate model for protein–dodecyl sulfate complexes, in which case, larger molecular weight proteins would show differences in the model for different hydrodynamic treatments.

In order to determine whether the observed differences for bovine serum albumin and ovalbumin were due to reoxidation during dialysis or not, a separate experiment was performed; 1% bovine serum albumin in 3% dodecyl sulfate and 1% β-mercaptoethanol was heated for 30 min at 90°. Equal aliquots were dialyzed against 0.1% dodecyl sulfate in distilled water and 0.1% dodecyl sulfate and 1.0% β-mercaptoethanol in distilled water. The measured relaxation times for the two aliquots after dialysis were the same as the values reported in Table II. Therefore, reoxidation during dialysis did not occur.

Since only two proteins showed differences in dimensions obtained by the two methods, an additional protein was treated and measured as discussed in the Experimental Section. Human transferrin was chosen because its molecular weight is of the same order of magnitude as that of bovine serum albumin. The relaxation times and calculated dimensions for this protein are shown in Tables II and III. Although no comparison may be made with these dimensions, the values are reasonably close to those for bovine serum albumin determined by transient birefringence.

The molecular weights of the proteins used in this study have also been examined by dodecyl sulfate gel electrophoresis on both 5 and 10% gels. These data are shown in Figure 1. As indicated, the molecular weights, as estimated on both 5 and 10% gels for the larger proteins, agree well with their known molecular weights; however, there are considerable differences for the lower molecular weight proteins.

Such variations between molecular weights as determined by dodecyl sulfate gel electrophoresis and known molecular weights of proteins have been noted by other investigators (Shapiro et al., 1967; Williams and Gratzer, 1971).

Additionally, dodecyl sulfate gel electrophoresis was used as a method of testing the homogeneity of the protein samples used in this study. Single bands were observed for each protein and therefore polydispersity may be ruled out as a cause for the two relaxation times observed for each protein.

## Discussion

The theoretical basis for determining molecular weights of polypeptide chains in dodecyl sulfate on polyacrylamide gels, proposed by Reynolds and Tanford (1970), has been supported in part by the hydrodynamic method of transient electric birefringence. This is apparent by the agreement between the dimensions of prolate ellipsoid models for dodecyl sulfate complexes with cytochrome *c*, lysozyme, β-lactoglobulin, and chymotrypsinogen A, calculated by the method reported here and the values reported by Reynolds and Tanford. The differences between dimensions of such models for ovalbumin and bovine serum albumin complexes with dodecyl sulfate, reported here and by Reynolds and Tanford, may be due to an inherent difference between the two hydrodynamic techniques or may be due to the assumption that protein-dodecyl sulfate complexes are rigid prolate ellipsoids. The agreement between the two techniques for the four lower molecular weight proteins tends to rule out the former reason for the observed differences. In support of the latter reason is the fact that two relaxation times were observed for all complexes studied, which is in agreement with transient birefringence theory for prolate ellipsoids (Ridgeway, 1966).

Intrinsic viscosity is determined under conditions in which a steady-state velocity gradient is maintained. If protein–dodecyl sulfate complexes were not rigid, then the complexes would appear more elongated in response to the steady-state shear forces acting during measurements of viscosity. In contrast, the relaxation time is a measure of the time of decay from a perturbed to an unperturbed state (Bur and Fetters, 1973). As such, measurements of transient birefringence are made under orienting force free conditions, and may reflect the undistorted shape of a deformable prolate ellipsoid. The lack of rigidity would become more apparent with increasing molecular weight.

A deformable prolate ellipsoid model for protein–dodecyl sulfate complexes is in agreement with the "necklace model" proposed by Shirahama et al. (1974). Their model is statistically elongated, with elongation increasing with molecular weight of the protein polypeptide. Such a necklace model is deformable, and the average structure may be the same as the deformable prolate ellipsoid model reported in this study. Such a model is in agreement with the results of Reynolds and Tanford, Shirahama et al., and the present study.

An interesting and important result of this study is the observation that the equivalent volume and axial ratio of a deformable particle do not remain the same under different experimental conditions which exist for the different hydrodynamic methods. Bradbury (1970) discussed three problems which are associated with the hydrodynamic theory of Scheraga and Mandelkern (1953). One of these problems is that the assumption must be made that the equivalent volume and axial ratio remain the same under the different hydrodynamic conditions which exist under the different experimental methods applicable to the theory of Scheraga and Mandelkern. Therefore, the problem discussed by Bradbury has been demonstrated to exist in fact, and one must take care when combining hydrodynamic parameters obtained by different experimental methods in order to determine hydrodynamic shape of macromolecules.

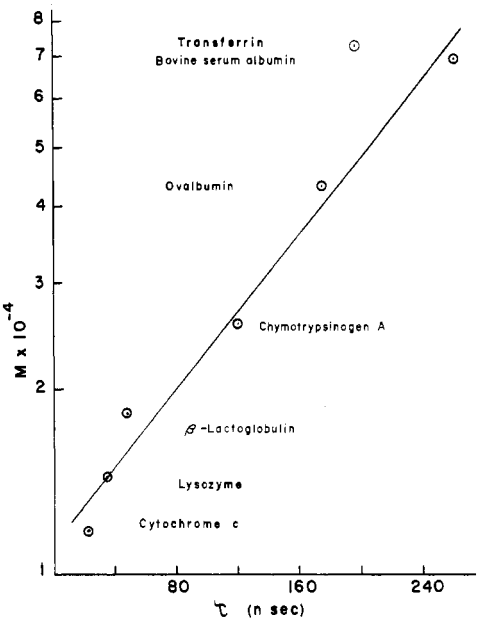


FIGURE 2: Empirical relationship for log $M$ vs. slow birefringence relaxation time for seven standard proteins.

Figure 2 shows an empirical linear relationship between the logarithm of the molecular weight of the protein moiety and the slow birefringence relaxation time for the protein–dodecyl sulfate complex. It is apparent from these data that there is some relation between the slow relaxation times and the molecular weight of the proteins. Therefore, protein molecular weights may be determined from measurements of the transient electric birefringence of protein–dodecyl sulfate complexes. The large departure of the transferrin point from the empirical line of Figure 2 is not understood. As discussed in the Results section, this sample was run several weeks after samples 1 through 6 were run. Although this may account for some variation, it does not explain such a large variation. Additionally, transferrin is a glycoprotein, which yields erroneously high molecular weights by sodium dodecyl sulfate gel electrophoresis (Leach and Fish, 1974). Uncertainty in molecular weight in the presence of dodecyl sulfate or anomalous dodecyl sulfate binding may account for the observation.

The rotary diffusion coefficient, $R_1$, for a long prolate ellipsoid with semi-axes $a$ and $b$ ($a > b$) is given by Scheraga et al. (1951) as

$$R_1 = (3kT/8\pi na^3)[\ln(2a/b) - 0.5] \quad (1)$$

According to the model suggested by Reynolds and Tanford (1970), $b$ is a constant and $a$ is proportional to the molecular weight, $M$, of the protein moiety of the dodecyl sulfate complex. Since $R_1$ is inversely proportional to the long relaxation time, $\tau_s$, we may expect the following relationship:

$$\tau_s \alpha M^3/(\ln M - 8.4) \quad (2)$$

The constant terms which appear in eq 1 have been evaluated in the proportionality statement (eq 2) according to

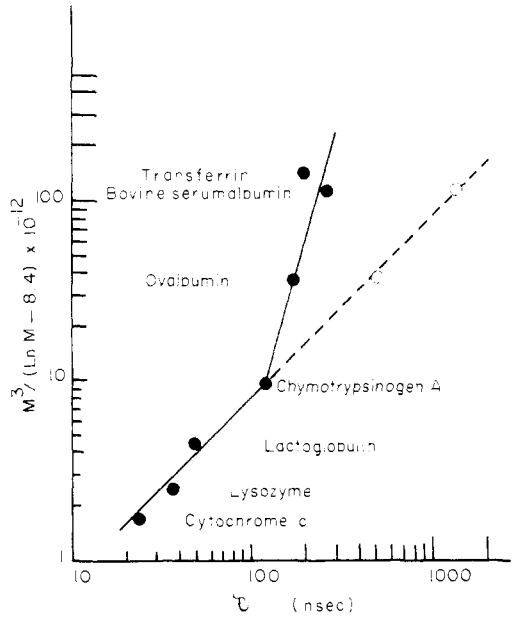


FIGURE 3: Theoretical relationship between molecular weight and slow birefringences relaxation time according to eq 2.

the proposed model of Reynolds and Tanford (1970).

The proportionality of statement 2 is not altered by taking the logarithm of both sides, which is shown in Figure 3. The solid circles in this figure represent the data of this experiment, and the open circles represent calculated relaxation times based on the dimensions reported by Reynolds and Tanford (1970) for ovalbumin and bovine serum albumin. The departure from linearity in the neighborhood of chymotrypsinogen A reflects lack of rigidity in the Reynolds and Tanford model. The fact that a second region of linearity results from such a plot of the data supports an ellipsoidal model. Thus, a deformable ellipsoidal model adequately explains the behavior shown in Figure 3. Figure 3 also provides a theoretical basis for molecular weight determination of proteins by the method of transient electric birefringence. It is interesting that the three proteins of lower molecular weight are resolved more distinctly by the transient birefringence method than by the gel electrophoresis method, as a comparison of Figures 1 and 3 shows.

The long relaxation times reported in this study are consistent with the observations of Rowe and Steinhardt (1974) that the rotational diffusion coefficients for protein-dodecyl sulfate complexes are considerably smaller than those of the native proteins, determined by transient birefringence. In agreement with the proportionality statement (eq 2), they found the rotational diffusion coefficients for such complexes, dependent on the negative third power of the length of the major semi-axis in the case of rods, to decrease with molecular weights of the protein moiety. Only one relaxation time was detected by Rowe and Steinhardt and this corresponds to the slow relaxation time reported here. For bovine serum albumin, ovalbumin and chymotrypsinogen A, their measured values agree with the values reported in Table II (E. S. Rowe, personal communication). The resolution of their instrument was 40 nsec, which may account for their failure to detect the fast relaxation process.

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