# Dynamic Viscoelastic Properties of Solutions of Paramyosin and Bovine Serum Albumin

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*The dynamic storage ($G'$) and loss ($G''$) shear moduli have been measured for solutions of paramyosin and bovine serum albumin in glycerol–water mixtures, using the apparatus of Birnboim and Ferry in the frequency range from 0.04 to 400 c.p.s., at temperatures between −20 and 10°. The paramyosin was studied in 70 and 80% glycerol with 0.6 M KCl and 0.6 M glycine at concentrations from 0.33 to 1%. At the lowest concentration, the viscoelastic properties conformed rather well to the Cerf–Scheraga theory for long prolate ellipsoids and can be ascribed to rigid-body orientation of the molecules. At higher concentrations, deviations appeared; when the glycine was omitted from the solvent, these deviations were greater and were associated with some loss in helical content as indicated by optical rotatory dispersion. The bovine serum albumin was studied in 88 to 93.5% glycerol at concentrations from 4 to 11%. The native protein showed no detectable viscoelasticity in the frequency range covered—merely Newtonian viscosity. However, after contact with the concentrated glycerol solvent for 3 days at room temperature, or upon acidification with 1.28 mequiv. of HCl/g. of protein, viscoelasticity appeared. Each of these two modified albumins exhibited a characteristic frequency dependence of viscoelastic properties which did not correspond to the molecular theories for either rigid elongated molecules or flexible random coils, and is presumably associated with some kind of intramolecular flexibility.*

## Introduction

A dilute solution of elongated, rigid macromolecules should exhibit viscoelastic properties when subjected to oscillating deformations; the frequency dependence predicted by the theories of Kirkwood and Auer for rods[1] and Cerf[2] and Scheraga[3] for prolate ellipsoids involves a single relaxation time, corresponding to the rotary diffusion of the molecule about an axis perpendicular to its long direction. Earlier measurements on solutions of polymerized fibrinogen[4] and helical poly-γ-benzyl-L-glutamate[5] have been compared qualitatively with the Kirkwood–Auer theory but did not agree closely with its predictions.

We now describe some dynamic viscoelastic studies of paramyosin and bovine serum albumin under various conditions of concentration and solvent medium. The solvents all contain large proportions of glycerol to achieve the high viscosities necessary for appropriate measurements in the available frequency range. For the most dilute paramyosin solution, the frequency dependence of the viscoelastic properties conforms rather well to the Cerf–Scheraga theory as reported in a recent note.[6] For the others, there are deviations to varying degrees which can be tentatively ascribed to intermolecular interference and intramolecular flexibility.

## Theory

The Kirkwood–Auer formulation, in terms of the reduced dimensionless storage modulus $G_R'$ and the corresponding solute contribution to the loss modulus, $(G'' - \omega v_1\eta_s)_R$, has been given previously.[5] The corresponding formulation of the Cerf–Scheraga theory differs only in the substitution of $^2/_5$ for the term $^1/_3$ in eq. 2 of ref. 5.

$$G_R' = \omega^2\tau^2/(1 + \omega^2\tau^2) \quad (1)$$

$$(G'' - \omega v_1\eta_s)_R = \omega\tau[1/(1 + \omega^2\tau^2) + {}^2/_5] \quad (2)$$

Here, $\omega$ is the circular frequency, $\tau$ the relaxation time, $v_1$ the volume fraction of solvent, and $\eta_s$ the solvent viscosity. These functions are plotted logarithmically against $\omega\tau$ in Figure 1, together with the corresponding Zimm theory[7] for flexible coils for comparison. Since the inflection in $G'' - \omega v_1\eta_s$ in most of the experimental data to follow fits the curve for ellipsoids slightly better than that for rods, we have used the former throughout although no conclusive distinction can be made. Following our usual procedure, the theoretical logarithmic curves are matched to the data by suitable horizontal and vertical adjustments. Since $G'/G_R' = (G'' - \omega v_1\eta_s)/(G'' - \omega v_1\eta_s)_R = 3cRT/5M$, where $c$ is concentration in g. of solute/ml. and $M$ is molecular weight, this amounts to determining $M$ and $\tau$ as adjustable parameters. The value of $M$ can be compared with that from other sources and $\tau$ can be compared with the calculated value for a prolate ellipsoid

$$\tau = \pi\eta_s L^3/9kT(2 \ln 2p - 1) \quad (3)$$

where $L$ is the ellipsoid length and $p$ is its axial ratio.

## Experimental Section

*Materials.* The paramyosin was extracted from the adductor muscle of the clam *Venus mercenaria* as described by Riddiford and Scheraga,[8] and was freeze-dried from a solution containing about 0.017 g. of protein/ml. with 0.6 $M$ potassium chloride and 0.05 $M$ potassium phosphate buffer at pH 7.0. It was stored in this form at 3°. The protein content of the stock powder was determined on the basis of light absorption in solution at 277 mμ, with the extinction coefficient

(1) J. G. Kirkwood and P. L. Auer, *J. Chem. Phys.*, **19**, 281 (1951).
(2) R. Cerf, *Compt. rend.*, **234**, 1549 (1952).
(3) H. A. Scheraga, *J. Chem. Phys.*, **23**, 1526 (1955).
(4) J. D. Ferry and F. E. Helders, *Biochim. Biophys. Acta*, **23**, 569 (1957).
(5) N. W. Tschoegl and J. D. Ferry, *J. Am. Chem. Soc.*, **86**, 1474 (1964).
(6) J. W. Allis and J. D. Ferry, *Proc. Natl. Acad. Sci. U. S.*, **54**, 369 (1965).
(7) B. H. Zimm, *J. Chem. Phys.*, **24**, 269 (1956).
(8) (a) L. M. Riddiford and H. A. Scheraga, *Biochemistry*, **1**, 95 (1962); (b) *ibid.*, **1**, 108 (1962).

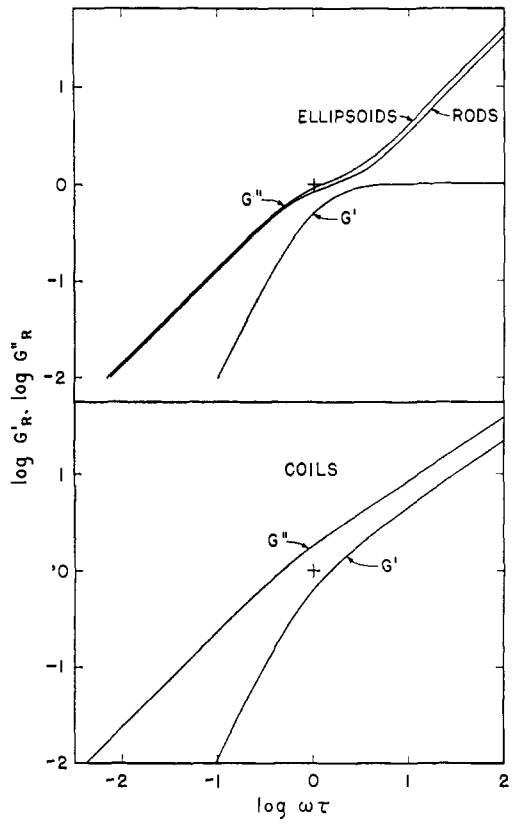


Figure 1. Logarithmic plots of the contributions of a macromolecular solute to the storage ($G'$) and loss ($G''$) components of the complex shear modulus, as predicted by the theories of Kirkwood and Auer (rods), Cerf and Scheraga (ellipsoids), and Zimm (coils).

used by Riddiford and Scheraga.[8] The intrinsic viscosity measured at pH 7.4 in 0.6 *M* potassium chloride and 0.06 *M* phosphate buffer was 2.21 dl./g., intermediate to the values of 1.92 dl./g. reported by Lowey[9] and 2.40 dl./g. by Kay.[10]

The bovine serum albumin was a crystalline preparation, lot W69204, obtained from the Armour Pharmaceutical Company. Its moisture content was determined to be 5.2% by loss of weight *in vacuo* at 85°, and the remainder was assumed to be pure protein in making up solutions subsequently from the stock preparation, even though the light absorption in solution at 279 mμ, using the extinction coefficient quoted by Foster,[11] indicated that only 90% rather than 94.8% of the stock was protein. The pH at a protein concentration of 1 g./dl. in 0.5 *M* potassium chloride was 5.7, close to the isoionic point. The sedimentation coefficient was determined in the solvent at several different concentrations, using a Spinco Model E ultracentrifuge,[12] and was found to follow the empirical equation

$$s = 4.65 - 0.58c \qquad (4)$$

where $s$ is in svedbergs (s.) at 25° in the designated solvent and $c$ is concentration in g./dl.; this is in substantial agreement with the results of Loeb and Scheraga.[13]

(9) S. Lowey, J. Kucera, and A. Holtzer, *J. Mol. Biol.*, 7, 234 (1963).
(10) C. Kay, *Biochim. Biophys. Acta*, 27, 469 (1958).
(11) J. F. Foster and K. Aoki, *J. Am. Chem. Soc.*, 80, 5215 (1958).
(12) We are indebted to Professor J. W. Williams for the use of this instrument.

The glycerol used to increase the solvent viscosity was obtained from Matheson Coleman and Bell or from Mallincrodt; the glycerol contents of these products were 99 and 95%, respectively, calculated from their densities[14] on the assumption that the only impurity was water.

*Preparation of Solutions and Solvents.* The paramyosin solutions contained water, glycerol, 0.6 *M* potassium chloride, and in some cases 0.6 *M* glycine, in addition to the small amount of phosphate buffer associated with the dry protein. Components were added in the following order: dry protein, water, glycine if required, and glycerol in which had been dissolved previously the potassium chloride necessary to supplement that present with the dry protein to bring the total concentration to 0.6 *M*. The glycerol content of each solution is expressed as the weight percentage of glycerol in the glycerol–water solvent exclusive of all solutes; the potassium chloride and glycine, as molarities referred to the glycerol–water mixture; and the protein, as weight percentage of the entire mixture.

The bovine serum albumin solutions contained water, glycerol, and in some cases hydrochloric acid in an amount (1.28 mequiv./g. of protein) which, in the absence of glycerol, would have decreased the pH to 2.7. Components were added in the following order: water necessary in addition to that contained in the protein; a portion of glycerol amounting to about half of the water; protein; the remaining glycerol, in two portions, while stirring with a magnetic stirrer at 3°, usually for several days. When acid was required, concentrated hydrochloric acid was premixed with the glycerol moiety and correspondingly less water was introduced at the beginning of the mixing procedure. In this case the solution could not be stirred more than 1 or 2 days at 3° without occurrence of time-dependent changes in properties.

For each solution, the corresponding solvent was made up without protein. It was necessary to have solvent density and viscosity data over a range of temperatures. Above 88% glycerol, the densities of glycerol–water mixtures were obtained from literature data. At lower glycerol concentrations, and for solvents containing potassium chloride or potassium chloride and glycine, the densities were measured pycnometrically. The viscosities of all the solvents were measured, usually by capillary viscometers,[15] but sometimes in the Birnboim apparatus (see below) as the low-frequency limit of the ratio of loss modulus to frequency; and for one solution by the falling-sphere method with miniature spheres of synthetic ruby.

As explained below, the viscoelastic measurements at various temperatures were reduced to reference temperatures of 0° for paramyosin and −10° for bovine serum albumin. The densities and viscosities of the relevant solvents at these reference temperatures are given in Table I. Complete data at all temperatures of measurement are tabulated elsewhere.[16] In the

(13) G. I. Loeb and H. A. Scheraga, *J. Phys. Chem.*, 60, 1633 (1956).
(14) C. Miner and N. Dalton, "Glycerol," Reinhold Publishing Corp., New York, N. Y., 1953; L. Bosart and A. Snoddy, *Ind. Eng. Chem.*, 19, 506 (1927).
(15) We are indebted to Mr. James F. Sanders for some of these measurements.
(16) J. W. Allis, Ph.D. Thesis, University of Wisconsin, 1965.

acidified solutions, the hydrochloric acid was assumed to have no effect on either the viscosity or the density of the solvent; it was, of course, mostly combined with the protein, and in any case presence of dilute HCl in 90% glycerol was found to have no significant effect, as shown in the table.

**Table I.** Densities and Viscosities of Solvents at Reference Temperatures

| Solvent composition | | | | | |
|---|---|---|---|---|---|
| Glycerol, % | KCl, M | Glycine, M | Temp., °C. | ρ, g./ml. | η, poise |
| 70 | 0.6 | 0.6 | 0 | 1.2244 | 0.87 |
| 80 | 0.6 | 0.6 | 0 | 1.2498 | 3.66 |
| 80 | 0.6 | ... | 0 | 1.238 | 3.10 |
| 88 | ... | ... | −10 | 1.250 | 28.3 |
| 90 | ... | ... | −10 | 1.256 | 46.9 |
| 90[a] | ... | ... | −10 | 1.260 | 47.3 |
| 91.5 | ... | ... | −10 | 1.260 | 63.9 |
| 92.5 | ... | ... | −10 | 1.262 | 80.0 |
| 93.5 | ... | ... | −10 | 1.265 | 94.4 |

[a] Containing 0.17 M HCl.

The densities of the solutions, for which approximate values were needed in connection with the viscoelastic measurements, were calculated with sufficient accuracy from the solvent densities and the protein partial specific volumes (0.730 ml./g. for paramyosin, 0.734 for albumin).

*Methods.* The viscoelastic measurements were made with the apparatus of Birnboim and Ferry[17] with modifications which have been described in subsequent publications.[18,19] A gold-plated cell[5] was used; for the acidified albumin solutions and some of the others, the oscillating rod was also gold-plated, but in other cases a stainless steel rod was used with no evidence of adverse effects. The storage and loss shear moduli were obtained in the frequency range from 0.04 to 400 c.p.s. Measurements were made at the reference temperature and at least one other, within the range from −20 to 10°.

Concomitant measurements of optical rotatory dispersion were made on a number of solutions. A Rudolph Model 200S spectropolarimeter[20] was employed, equipped with a Keithley photometer unit which allowed the voltage on the photomultiplier tubes to be adjusted between 300 and 1200 v. The temperature was 20 ± 0.1° and the wave length range from 320 to 680 mμ. Plots of $-[\alpha](\lambda^2 - \lambda_0^2)$ against $(\lambda^2 - \lambda_0^2)^{-1}$, with $\lambda_0 = 212$ mμ, were linear in almost every case with very little scatter, in accordance with the Moffitt equation.[21] Here $[\alpha]$ is the specific rotation at wave length λ. The slope of this plot, multiplied by $100(n^2 + 2)\lambda_0^4/3M_0$, where $n$ is the refractive index of the solvent and $M_0$ the mean residue weight of the protein, gives the constant $b_0$ of the Moffitt equation, which can be correlated with helical content.[22] The values of $n$ for the solvents concerned were measured with an Abbé refractometer, and $M_0$ was taken as 119 for paramyosin and 115 for the albumin.

(17) M. H. Birnboim and J. D. Ferry, *J. Appl. Phys.*, **32**, 2305 (1961).
(18) R. B. DeMallie, M. H. Birnboim, J. E. Frederick, N. W. Tschoegl, and J. D. Ferry, *J. Phys. Chem.*, **66**, 536 (1962).
(19) N. W. Tschoegl and J. D. Ferry, *Kolloid-Z.*, **189**, 37 (1963).
(20) We are indebted to Professor Robert A. Alberty for the use of this instrument.
(21) W. Moffitt and J. T. Yang, *Proc. Natl. Acad. Sci. U. S.*, **42**, 596 (1956).
(22) H. A. Scheraga, "Protein Structure," Academic Press Inc., New York, N. Y., 1961, pp. 177–191.

## Paramyosin. Results and Discussion

*Solutions Containing Glycine.* Glycine was included in the mixed solvent for paramyosin to attempt to improve the stability by raising the dielectric constant, after some loss of helical content had been noted in the solvent glycerol–water–potassium chloride (see below). It proved to be impossible to measure optical rotatory dispersion directly in the mixed solvent with glycine, since the solvent itself exhibited rotation. However, three such solutions were dialyzed after viscoelastic measurements into aqueous 0.6 M potassium chloride–0.06 M phosphate buffer at pH 7.4, with three changes of large volumes of dialyzate at intervals of at least 8 hr., and the resulting aqueous solutions were subjected to rotatory dispersion measurements. The values of $b_0$ ranged from −617 to −641. A similar series of measurements on paramyosin dissolved directly in aqueous 0.6 M potassium chloride–0.01 M phosphate buffer at pH 7.4, without previous exposure to glycerol, gave $b_0 = -617$. These values may be compared with −600 found by Simmonds, *et al.*,[23] in 0.6 M potassium chloride–0.01 M phosphate buffer at pH 7.4, and −570 found by Riddiford and Scheraga[8] in the same buffer with 0.3 M potassium chloride; they indicate essentially 100% helical content. The exposure to glycerol plus glycine thus produced no irreversible changes, at least, in the secondary protein structure.

Viscoelastic measurements were made at 0.33% protein in 70% glycerol with 0.6 M KCl and 0.6 M glycine at −20.2 and 0°, and in 80% glycerol–KCl–glycine at 0 and 10.0°. All data were reduced to 0° by the usual method of reduced variables,[18,19] taking $a_T = (\eta - \nu_1\eta_s)T_0\rho_0/(\eta - \nu_1\eta_s)_0T\rho$, where $\eta$ and $\rho$ are the solution viscosity and density, and the subscript 0 refers to the reference temperature. After reduction, logarithmic plots of $G'$ and $G'' - \omega\nu_1\eta_s$ were matched to the Cerf–Scheraga theory, determining log $\tau$ to be −2.31 and −1.84, respectively. Thus $\tau$ is not quite directly proportional to $\eta_s$ (comparing the two solvents, $\Delta \log \tau = 0.47$, $\Delta \log \eta_s = 0.62$). However, when the data for both solutions are plotted logarithmically against $\omega\tau$, as shown in Figure 2, they agree very closely, showing that the frequency dependence of $G'$ and $G''$ has the same form in 70 and 80% glycerol. The agreement with the theoretical curve for $G''$ is very close, and that for $G'$ is quite good, representing the best conformity to the theory for elongated rigid particles that has thus far been observed.

According to the phenomenological theory of linear viscoelasticity, a slope of unity for log $G''$, or log $(G'' - \omega\nu_1\eta_s)$, at high frequencies requires that $G'$ be frequency independent as the Cerf–Scheraga theory does prescribe; the slopes are connected by the relation[24]

$$\text{d log } G'/\text{d log } \omega = F(G''/G')(1 - \text{d log } G''/\text{d log } \omega) \quad (5)$$

(23) N. S. Simmonds, C. Cohen, A. G. Szent-Gyorgyi, D. B. Wetlaufer, and E. R. Blout, *J. Am. Chem. Soc.*, **83**, 4766 (1961).
(24) J. D. Ferry, "Viscoelastic Properties of Polymers," John Wiley and Sons, Inc., New York, N. Y., Chapter 4, eq. 9 and 20.

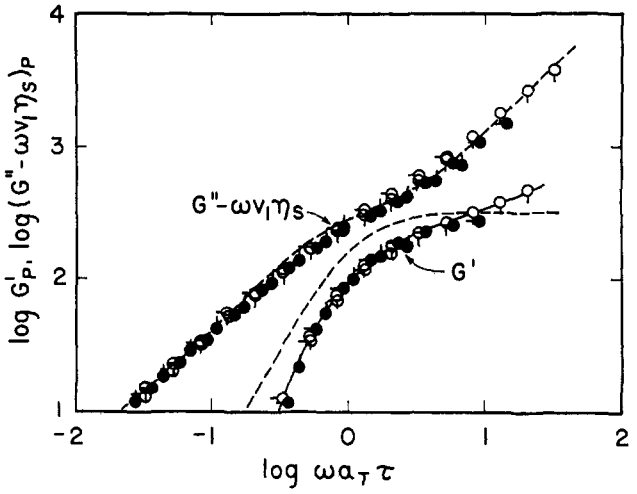


Figure 2. Logarithmic plots of $G'$ and $G'' - \omega v_1 \eta_s$ for 0.33% paramyosin in 70% glycerol (open circles) and 80% glycerol (closed circles), with 0.6 $M$ KCl and 0.6 $M$ glycine, against $\omega\tau$. All data were reduced to 0°. Temperatures of measurement: pip down, −20.2°; left, 0.0°; up, 10.0°. Subscript p denotes multiplication by $T_0\rho_0/T\rho$.. Dashed lines represent Cerf-Scheraga theory with parameters in Table II.

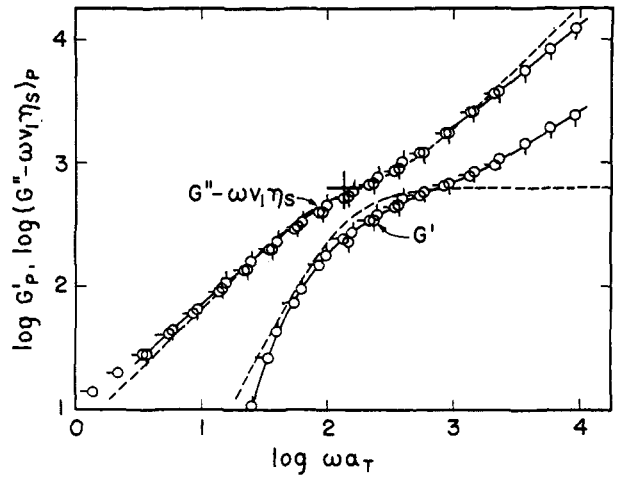


Figure 3. Logarithmic plots of $G'$ and $G'' - \omega v_1 \eta_s$ for 1.0% paramyosin in 70% glycerol, with 0.6 $M$ KCl and 0.6 $M$ glycine, against $\omega$. Temperature key: pip down, −20.2°; left, −10.0°; up, 0.0°. All data are reduced to 0°. Dashed lines represent Cerf-Scheraga theory.

where $F$ is a factor not far from unity. However, there is no inconsistency between the fairly close adherence of $G'' - \omega v_1 \eta_s$ to a line of unit slope in Figure 2 and a significant positive slope for $G'$, since in this region $G''$ (including the solvent contribution $\omega v_1 \eta_s$) exceeds $G'$ by about a factor of ten. Thus, the slope of $G'$ is much more sensitive to additional relaxation mechanisms than is the deviation of the slope of $G''$ from unity. These mechanisms may arise from a slight flexibility of the long molecule.

Measurements were made also at 0.50% protein in 80% glycerol–KCl–glycine and 1.00% protein in 70% glycerol–KCl–glycine. The data for the latter are plotted in Figure 3. With increasing protein concentration, the slope of $G'$ at high frequency increases and that of $G'' - \omega v_1 \eta_s$ begins to deviate perceptibly from unity. The data are matched to the Cerf-Scheraga theory by adjusting the ordinate and abscissa scales, the cross corresponding to the origin of Figure 1.

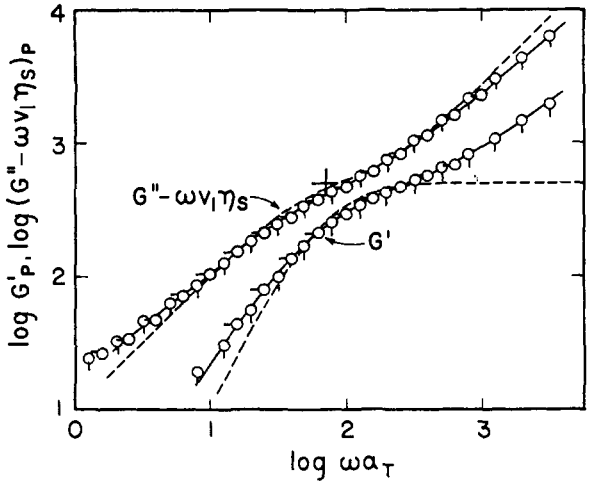


Figure 4. Logarithmic plots of $G'$ and $G'' - \omega v_1 \eta_s$ for 1.0% paramyosin in 80% glycerol with 0.6 $M$ KCl, reduced to 0°: pip down, −10.0°; left, 0.0°. Dashed lines represent Cerf-Scheraga theory.

*Solutions without Glycine.* Optical rotatory dispersion measurements were made on a 1% solution in 80% glycerol with 0.6 $M$ potassium chloride and no glycine, and also on this solution after the glycerol had been dialyzed out as described above. The values of $b_0$ were −354 (or, corrected[8b,25] to the refractive index of the glycerol-free solvent, −398) and −524, respectively. These indicate a substantial loss of helical character in glycerol without the stabilizing influence of the glycine, though it appears to be partially recovered after removal of the glycerol. Viscoelastic measurements on a similar solution are shown in Figure 4. The fit to the Cerf–Scheraga theory is slightly poorer. Viscoelastic measurements were also made on a 0.46% solution in the same solvent, with similar results.

*Parameters from the Cerf–Scheraga Theory.* From the match of theory to experiment in Figures 3 and 4 and similar plots for the other solutions, values of $M$ and $\tau$ are obtained. In Table II, these are compared with the molecular weight of 220,000 reported by Lowey[9] and the relaxation time calculated by eq. 3 taking $L$ = 1390 Å. and $p$ = 69.5. The apparent molecular weights increase slightly with protein concentration and are in reasonable agreement with the value of Lowey, though lower than that concluded by Riddiford and Scheraga[8] (log $M$ = 5.52). The fact that the viscoelastic molecular weight has the right magnitude is further strong confirmation of the validity of the Cerf–Scheraga theory. The relaxation times are all somewhat larger than those calculated for infinite dilution, and the discrepancy increases with concentration. This is reasonable since even at 0.33% there will be some intermolecular interference in the rotary diffusion of this highly elongated molecule. In the absence of glycine, the slope of $G'$ at high frequencies is somewhat higher, suggesting a greater flexibility associated with diminished helical content.

(25) J. A. Schellman, *Compt. rend. trav. lab. Carlsberg*, 30, 363 (1958).

Table II. Parameters from Viscoelastic Measurements on Paramyosin (0.6 $M$ KCl, 0°, Log $M$ = 5.35)

| Protein concn., % | Glycerol concn., % | Glycine concn., $M$ | Log $M$, obsd. | Log $\tau$, obsd. | Log $\tau$, calcd. | d log $G'$/ d log $\omega^a$ | Log $J_e$ |
|---|---|---|---|---|---|---|---|
| 0.33 | 70, 80 | 0.6 | 5.25 | −1.84[b] | −1.97 | $0.3_5$ | −3.5 |
| 0.50 | 80 | 0.6 | 5.40 | −1.61 | −1.97 | $0.4_5$ | −3.4 |
| 1.00 | 70 | 0.6 | 5.42 | −2.14 | −2.59 | 0.6 | −3.3 |
| 0.46 | 80 | 0 | 5.40 | −1.90 | −1.97 | 0.5 | −2.8 |
| 1.00 | 80 | 0 | 5.53 | −1.86 | −1.97 | 0.7 | ...[c] |

[a] At high frequency. [b] Corresponding to 80% glycerol. [c] $G'$ not closely proportional to $\omega^2$ at low frequencies.

Independently of any molecular theory, the steady-state compliance, $J_e$, a measure of the elastic energy stored in steady-state flow, may be calculated from the viscoelastic measurements at low frequencies where $G'$ is proportional to $\omega^2$ and $G''$ is proportional to $\omega$; $J_e = (dG'/d\omega^2)_{\omega\to 0}/\eta^2$. In Table II, log $J_e$ is also given (units cm.²/dyne). In very dilute solutions, this quantity is expected[26] to be directly proportional to concentration; it does increase somewhat with concentration, and it is also increased by the exposure to glycerol without glycine.

## Bovine Serum Albumin. Results and Discussion

*Isoelectric Solutions.* The essentially isoelectric protein, without added acid, was subjected to viscoelastic measurements at a concentration of 7% in 93.5% glycerol, 8% in 92.5% glycerol, 9% in 91.5% glycerol, and 11% in 90% glycerol. All these solutions were maintained at 3° during mixing and exposed to room temperature only briefly while loading the apparatus. The measurements were made at −10 and −19°, and the data were reduced to −10°. In every case, only the loss component of the modulus was measured, $G'$ being too small to detect. The results for the most concentrated solution are shown in Figure 5. The solute contribution to the loss modulus is directly proportional to the frequency, and thus the experiment merely provides an unusually elaborate means of measuring the viscosity. The viscosities at −10° for the four solutions listed above were 184, 169, 129, and 124 poises, respectively.

It is puzzling that no elasticity is detected, since log $\tau$, calculated from eq. 3 with $L$ = 190 Å. and $p$ = 5 in 90% glycerol at −10°, is −3.1. As seen in Figures 2–4, the storage modulus associated with rigid ellipsoid orientation should appear at somewhat lower frequencies than $1/\tau$ and therefore within the frequency range of Figure 5. The length used for this calculation is based primarily on flow birefringence measurements by Edsall and Foster[27] which involve essentially the same hydrodynamic orienting forces as in our investigations and were made at a not very different concentration (4.5 g./dl., as compared with 7 to 11% for our isoelectric solutions). Our somewhat higher concentrations should, if anything, give a longer relaxation time.

*Aged Isoelectric Solutions.* In other solutions of the essentially isoelectric albumin, the mixing was done at room temperature and 3 days elapsed between the exposure to glycerol and the viscoelastic measurements. No adverse effect was expected, since rotary diffusion constants (corrected for solvent viscosity) and molecular dimensions obtained by Edsall and Foster[27] from flow birefringence in 88.5% glycerol agree well with those obtained by Krause and O'Konski[28] from electrical birefringence in pure water. However, a striking difference in viscoelastic properties appeared as illustrated in Figure 6 for 8% protein in 92.5% glycerol. A substantial storage component $G'$ is now found and it is directly proportional to $\omega^2$ as expected at low frequencies. The data do not extend to high enough reduced frequencies for critical comparison with the Cerf–Scheraga theory; at low frequencies where $G'$ is proportional to $\omega^2$ and $G''$ to $\omega$, they can be fitted equally well to the Zimm theory for flexible coils (Figure 1). Similar measurements were made at protein concentrations of 4 and 6%. In each case, measurements made at two different temperatures showed good agreement after reduction to a reference temperature of −10°. The values of $M$ and $\tau$ from Cerf–Scheraga and Zimm fits at these concentrations are also given in Table III.

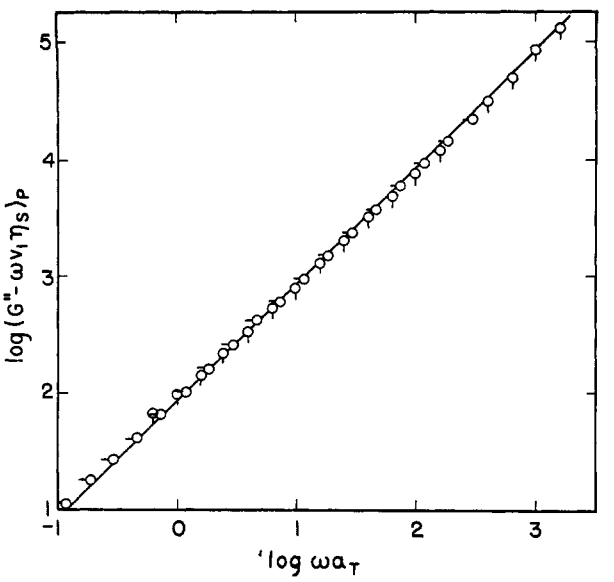


Figure 5. Logarithmic plot of solute contribution to loss modulus, $G'' - \omega v_1 \eta_s$, for 11% bovine serum albumin in 92.5% glycerol, reduced to −10.0°: pip down, −10.0°; left, 0.0°.

The development of an elastic component on aging 3 days at room temperature was not accompanied by any change in the optical rotatory dispersion. However, the sedimentation coefficient measured after dialyzing out the glycerol and diluting the protein concentration to 1.00 g./dl. was 4.32 s., which is about 6%

(26) K. Ninomiya and J. D. Ferry, in preparation.
(27) J. T. Edsall and J. F. Foster, *J. Am. Chem. Soc.*, 70, 1860 (1948).
(28) S. Krause and C. T. O'Konski, *ibid.*, **81**, 5082 (1959).

Table III. Parameters from Viscoelastic Measurements on Bovine Serum Albumin (−10.0°, Log $M$ = 4.83)

| Protein concn., % | Glycerol concn., % | mequiv. of HCl/g. of protein | Cerf-Scheraga Log $M$ obsd. | Cerf-Scheraga Log $\tau$ obsd. | Cerf-Scheraga Log $\tau$ calcd.[b] | Zimm Log $M$ obsd. | Zimm Log $\tau$ obsd. | Log $J_e$ |
|---|---|---|---|---|---|---|---|---|
| 4.0[a] | 92.5 | 0 | 4.01 | −3.37 | −2.75 | 4.58 | −3.22 | −6.0 |
| 6.0[a] | 92.5 | 0 | 4.27 | −3.14 | −2.75 | 4.85 | −2.97 | −5.8 |
| 8.0[a] | 92.5 | 0 | 4.55 | −2.98 | −2.75 | 5.15 | −2.80 | −5.5 |
| 5.0 | 92.5 | 1.28 | .. | .. | .. | 5.06 | −2.59 | −5.3 |
| 7.5 | 90 | 1.28 | .. | .. | .. | 6.01 | −1.73 | −4.4 |
| 9.0 | 88 | 1.28 | .. | .. | .. | 6.17 | −1.93 | −4.3 |

[a] Aged 3 days at room temperature. [b] Calculated from eq. 3 with the solvent viscosity.

higher than that of the native protein at the same concentration according to eq. 4.

*Acidified Solutions.* Another group of albumin solutions was made up containing 1.28 mequiv. of hydrochloric acid/g. of protein. In the absence of glycerol, this was sufficient to lower the pH to 2.7 which is in the range where molecular expansion occurs as deduced by various physical measurements.[29] A series of optical rotatory dispersion measurements at 1% concentration with different amounts of acid both in water and in aqueous glycerol, when analyzed in the manner of Leonard and Foster,[30] indicated that with 1.28 mequiv. of acid/g. of protein in glycerol the isomerization process postulated by these authors was largely accomplished but not the expansion.[31] Thus the glycerol appeared to inhibit the expansion stage of the acid-induced modification. Nevertheless, the modification was sufficient to produce an elastic response as illustrated in Figure 7 for 9% protein in 88% glycerol. Results at two different temperatures agree well after reduction; the frequency dependence, however, does not correspond very well to the theory for either ellipsoids or coils, though the theoretical Zimm curves for coils are drawn for an approximate match at low frequencies. Similar data on acidified solutions were obtained for 5% protein in 92.5% glycerol and 7.5% protein in 90% glycerol. Values of $M$ and $\tau$ obtained by matching to the Zimm theory at low frequencies are included in Table III.

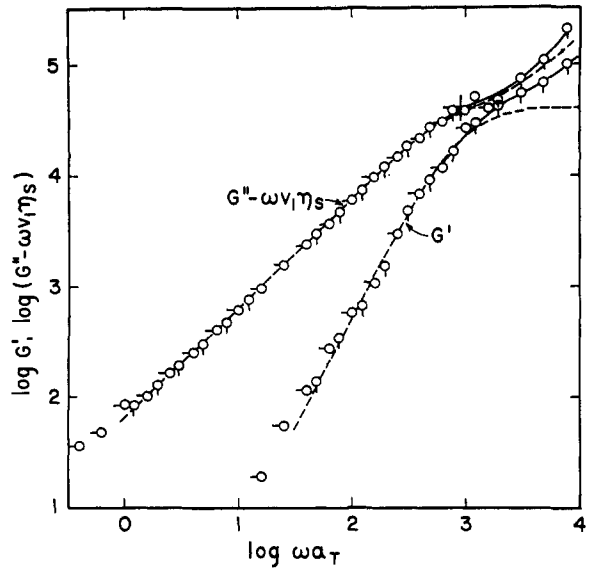


Figure 6. Logarithmic plots of $G'$ and $G'' - \omega v_1 \eta_s$ for 8% albumin in 92.5% glycerol, after standing 3 days at room temperature, reduced to −10.3°: pip down, −18.7°; left, −10.3°. Dashed lines represent Cerf-Scheraga theory.

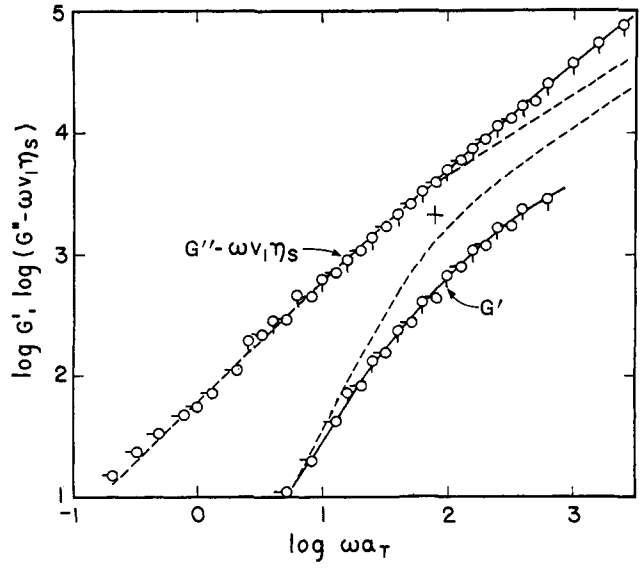


Figure 7. Logarithmic plots of $G'$ and $G'' - \omega v_1 \eta_s$ for 9% acidified albumin in 88% glycerol, reduced to −10.0°: pip down, −10.0°; left, 0.0°. Dashed lines represent Zimm theory.

*Parameters from Theory.* Since the native albumin molecule, at the rather high concentrations studied, does not exhibit the orientational viscoelasticity predicted by the Cerf-Scheraga theory, it is perhaps not surprising that the elasticity evoked by acid or aging in glycerol does not follow the Cerf-Scheraga theory either. For the aged isoelectric solutions, this conclusion cannot be definitely made from the frequency dependence (Figure 6), but it is evident from log $M$ and log $\tau$ in Table III; the Cerf-Scheraga molecular weights are all much too small, and the relaxation times are smaller even than the calculation on the basis of infinite dilution (solvent viscosity). Thus the elasticity is presumably derived not from rigid-body orientation but from some kind of intramolecular flexibility. Application of the Zimm theory to the glycerol-aged solutions gives more reasonable values for $M$ but its validity is doubtful. Matching the Zimm theory to the acidified solutions gives molecular weights which are much too high; in any case Figure 7 shows that the

(29) J. F. Foster in "The Plasma Proteins," F. W. Putnam, Ed., Academic Press Inc., New York, N. Y., 1960, Chapter 6.

(30) W. J. Leonard, Jr., and J. F. Foster, *J. Biol. Chem.*, 236, 2662 (1961).

(31) Details of these studies are given in ref. 16. With substitution of $H_2SO_4$ for HCl, expansion did not occur even in water, as judged by optical rotatory dispersion.

Zimm theory cannot be expected to be applicable. The acid-modified albumin is certainly not a random coil, though it may have internal viscoelastic responses in view of the flexibility revealed by fluorescence depolarization.[29] In principle, the viscoelastic properties of the molecule itself could be derived from those of the solution, though such a calculation has been formulated only for spherical molecules at infinite dilution.[32]

The steady-state compliance of the albumin solutions is also given in Table III. It is much smaller than for the paramyosin solutions but increases rapidly with protein concentration.

## Conclusions

In dilute paramyosin solutions, the viscoelastic behavior can be attributed to rigid-body orientation of the elongated molecules and can be described fairly well by the Cerf–Scheraga theory. Exposure to concentrated glycerol without glycine appears to increase the molecular flexibility slightly. In concentrated bovine serum albumin solutions, viscoelastic behavior appears only after molecular modification by aging in concentrated glycerol or by slight acidification. Here the viscoelasticity appears to arise primarily from intramolecular flexibility rather than rigid-body orientation. It cannot be described by any molecular theory at present. Such measurements may have promise for obtaining additional information about intramolecular flexibility in biological macromolecules.

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(32) R. Cerf, *J. Chem. Phys.*, 20, 395 (1952).

# Excimer Fluorescence in Liquid Phenol, *p*-Ethylphenol, and Anisole[1,2]

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*The fluorescence emission spectra of phenol, p-ethylphenol, and anisole have been investigated. An additional fluorescence band in the liquid state has been observed which was shifted 5200–5600 cm.$^{-1}$ to lower frequency from that found for the dilute solution band. This new band is due to the emission of excited dimers or excimers in distinction to the monomer band, the latter being due to the emission from excited monomers. From the temperature dependence of the emission of each band, the heat and entropy of activation for the photoassociation, $\Delta H_a$ and $\Delta S_a$, of phenol and anisole have been determined. For phenol $-\Delta H_a = 4.5 \pm 0.5$ kcal./mole and $\Delta S_a = -25 \pm 3$ cal./mole deg. For anisole $-\Delta H_a = 5.1 \pm 0.6$ kcal./mole and $\Delta S_a = -22 \pm 3$ cal./mole deg. The variation of the wave length of maximum fluorescence for these compounds in the liquid and solid states and in different solvents has been interpreted as being due to hydrogen bonding. This bonding appears to be stronger in the excited state than in the ground state.*

## Introduction

The appearance of a new emission band in concentrated solutions of certain aromatic hydrocarbons is due to photoassociation and subsequent emission at higher wave lengths by an excited dimer or excimer[4,5]

$$A^* + A \longrightarrow A_2^* \quad (1)$$

$$A_2^* \longrightarrow 2A + h\nu_D \quad (2)$$

This emission competes with the monomer fluorescence

$$A^* \longrightarrow A + h\nu_M \quad (3)$$

and monomer quenching processes

$$A^* \longrightarrow A \quad (4)$$

The excimer can also dissociate more readily at higher temperatures

$$A_2^* \longrightarrow A + A^* \quad (5)$$

and relax nonradiatively

$$A_2^* \longrightarrow 2A \quad (6)$$

to re-form the dissociated molecules in the ground state.

The probability of excimer formation is generally higher the longer the lifetime of the excited monomer, the more concentrated the solution, and the lower the temperature. In these systems, the transition from the $^1L_b$ excited singlet state to the $^1A$ ground state is associated with a weaker transition moment than the transition from the $^1L_a$ state. Since the emission occurs from the lowest excited state, a longer lifetime is apparent when the $^1L_b$ state is lower than the $^1L_a$ state.[5] Most of the compounds that have produced excimer emission have a lower $^1L_b$ state.

Excimer emission has been observed in the molten and crystalline states where, of course, the concen-

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(2) For the previous paper in this series, see A. Pesce, E. Bodenheimer, K. Norland, and G. D. Fasman, *J. Am. Chem. Soc.*, 86, 5669 (1964).

(3) This work was done during the tenure of an established investigatorship of the American Heart Association.

(4) Th. Förster and K. Kasper, *Z. Elektrochem.*, 59, 977 (1955).

(5) J. B. Birks and L. G. Christophorou, *Proc. Roy. Soc.* (London), A277, 571 (1964), and earlier references cited.