Anal. Calcd. for $C_{11}H_{11}NO_2$: C, 69.81; H, 5.86. Found: C, 69.97; H, 5.79.

From α-Benzoylaminocrotonic Azlactone.—Five grams of a mixture of the *cis* and *trans* forms of α-benzoylaminocrotonic azlactone[13] of m. p. 118–122° was hydrolyzed by refluxing for three hours with 200 ml. of 1 *N* hydrochloric acid.[3] After the solution had cooled, 3 g. of what was presumably a mixture of α-benzoylaminocrotonic and benzoic acids was removed by filtration. The filtrate was neutralized with 2 *N* sodium hydroxide, and a solution of 3.4 g. of *as*-methylphenylhydrazine in 10 ml. of water and 5 ml. of glacial acetic acid was added to it. After standing overnight the solution was chilled and the solid methylphenylhydrazone that had separated was collected and washed. It was suspended in a solution of 15 ml. of concentrated hydrochloric acid in 30 ml. of water, and the mixture was heated on the steam-bath with swirling for thirty minutes. The suspension was cooled and filtered, and the solid was dissolved in aqueous sodium hydroxide. After removal of oily impurities by ether extraction, the aqueous solution was boiled with charcoal, filtered and acidified. The acid that separated was collected, washed with water and dried at 60°; yield, 1.3 g. (29.4% from the azlactone). After three recrystallizations from benzene, the melting point of the acid (and of mixtures with the acid described in the preceding paragraph) was 215–216° (dec., lit.,[2] 213°). The infrared absorption spectra[14] of the samples obtained by the two different methods were identical (see the figures). Both samples separated from benzene solution as needles which in contact with the mother liquor soon changed into crystals of granular texture.

Decarboxylation of the above acid at 225° followed by distillation at 15 mm. (bath temperature 130–160°) yielded 1,3-dimethylindole, identified by its refractive index ($n^{20}D$ 1.5929), infrared absorption spectrum (see the figure), and the melting point and mixed melting point (142.5–143°) of the picrate. The sample used for comparison had been obtained by hydrolysis and decarboxylation of 1-methyl-3-indoleacetonitrile (II).[1]

1,3-Dimethyl-2-indolecarboxamide. From the Acid.—A suspension of 0.32 g. of the above acid (V) in 3.2 ml. of redistilled acetyl chloride was cooled in an ice-bath and 0.42 g. of phosphorus pentachloride was added. The mixture was swirled until homogeneous and then allowed to stand at room temperature for two and three-fourths hours. The solvent was removed *in vacuo* with the bath temperature not exceeding 45°. The solid residue was chilled and 10 ml. of ice-cold concentrated aqueous ammonia solution was added to it. The temperature was slowly raised to 72° over a period of thirty minutes with constant stirring. The suspension of the amide was then cooled and the solid was collected, washed with concentrated aqueous ammonia followed by water, and dried at 60°; yield, 0.28 g. (87.5%). After two recrystallizations from benzene–absolute alcohol the product melted at 213.5–214°. The mixed melting point with the amide obtained by hydrolysis of 1,3-dimethyl-2-cyanoindole (III) was 213–214°.

(13) The azlactone was kindly put at the authors' disposal by Dr. H. E. Carter.

(14) The authors are indebted to Mrs. Agatha Roberts Johnson for the absorption studies.

Summary

The reaction of the methiodide of 1-methyl-3-dimethylaminomethylindole with aqueous sodium cyanide affords, in addition to the normal alkylation product, a small amount of 1,3-dimethyl-2-cyanoindole. The structure of this product has been proved by conversion to the corresponding amide and acid which were identical with compounds obtained by independent syntheses.

URBANA, ILLINOIS RECEIVED JANUARY 2, 1948

[CONTRIBUTION FROM DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Studies on Double Refraction of Flow. IV. Human Serum γ-Globulin and Crystallized Bovine Serum Albumin[1]

BY JOHN T. EDSALL AND JOSEPH F. FOSTER[2]

In previous papers of this series, the molecular dimensions of zein[3] and of fibrinogen[4] have been studied by the method of double refraction of flow, the results being interpreted in the light of viscosity, sedimentation and other measurements. In the present study, we report results of similar investigations on human serum γ-globulin[5] and crystallized bovine albumin.[6] The orientation of these molecules, by means of a velocity gradient, to an extent sufficient for accurate double refraction measurements, required the use of solvents of high viscosity, in order to diminish the rotary Brownian movement. Whereas fibrinogen, with a molecular length near 700 Å., could be readily studied in 40% glycerol, γ-globulin required 60–76% glycerol, and serum albumin approximately 90% glycerol.

(1) This paper is Number 68 in the series "Studies on the Plasma Proteins" from Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry, and Number XVII in the series "Preparation and Properties of Serum and Plasma Proteins" from the same laboratory.

The preparations of serum globulin employed were prepared from blood collected by the American Red Cross, under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

(2) Present address, Department of Chemistry, Iowa State College, Ames, Iowa.

(3) J. F. Foster and J. T. Edsall, THIS JOURNAL, **67**, 617 (1945).

(4) J. T. Edsall, J. F. Foster and H. Scheinberg, *ibid.*, **69**, 2731 (1947).

(5) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, Jr., in preparation.

(6) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, *ibid.*, **69**, 1753 (1917).

Experimental Methods

The apparatus used[7] and the methods of measurement[3,4] have already been described in detail.

The great majority of the measurements on γ-globulin were made on a single preparation (IIGl-L371) prepared from Fraction II + III of human plasma[8] by method 3c as described by Oncley, *et al.*[5] Electrophoretically, this preparation contained 97% γ-globulin, 2% albumin, and 1% β-globulin. Such preparations, however, have been

(7) J. T. Edsall, C. G. Gordon, J. W. Mehl, H. Scheinberg and D. W. Mann, *Rev. Sci. Instruments*, **15**, 243 (1944).

(8) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

shown by Oncley, Scatchard and Brown[9] to be heterogeneous in the ultracentrifuge. The implications of this heterogeneity are further discussed later in this paper. One run not reported in detail here was made on preparation IIG1-120, with results essentially indistinguishable from others in the series.

The crystallized bovine albumin was from lot 27-315, prepared at the Armour Laboratories according to methods developed by Cohn and Hughes.[6]

The glycerol-water mixtures used as solvents were prepared by the method previously described for the studies on fibrinogen. The γ-globulin preparations contained a small amount of sodium chloride in addition to the glycerol-water used as solvent. No salt was added to the albumin preparations. At the very high glycerol concentrations employed in the studies of albumin (88.5–94.5% by weight) the viscosity varies rapidly with slight changes in the composition of the solvent. Therefore, great care was taken in the control of glycerol concentration. Both γ-globulin and albumin gave clear and stable solutions in the glycerol-water mixtures employed.

Experimental Results

γ-Globulin.—A series of measurements on γ-globulin preparation IIG1-L371 was made at protein concentrations varying from 1.25 to 5 g. per 100 cc., and at glycerol concentrations varying from 60 to 76%. In almost all of these experiments, studies were made at two different temperatures; one set near 6° and the other at 18 to 19°. The results of one experiment are given in detail in Table I, together with the calculated values of the rotary diffusion constants (Θ) derived from the measurements. The values of Θ are not reported as such, but are multiplied by the ratio of the viscosity of the solvent (η) to the absolute temperature (T), since these $\eta\Theta/T$ values should be independent of the particular solvent and temperature employed, as long as the molecules retain their shape. The values of the extinction angle, χ, in two experiments were so close to 45° that the probable error in the calculations is very large, and the resulting values of $\eta\Theta/T$ are given no weight. These values are enclosed in parentheses in the final column of Table I. The other values are all reasonably consistent, and lead to an over-all estimated $\eta\Theta/T$ of 22 poise per sec. per degree (see Table II). The double refraction measurements are reported in the second half of Table I. The measured phase differences are a linear function of the product ($G\eta$) of the velocity gradient and viscosity, at $G\eta$ values up to 4000 or above. The increase of phase difference at higher velocity gradients is slightly less than linear, as would be expected from theoretical considerations.

TABLE I

MEASUREMENTS OF χ AND Δ ON HUMAN γ-GLOBULIN PREPARATION IIG1-L371

Protein concentration 1.25%. Solvent: glycerol 76%, water 24% by weight. Viscosity of solvent (η) 0.93 at 6°, 0.49 at 18.7°.

Temp., °C.	Speed, R. P. M.	$G\eta$	χ	α	$\frac{\eta\Theta}{T}$
6.3	300	2850	44.3 ± 0.78	0.147	(70)
6.0	360	3500	43.0 ± .52	0.42	(30)
6.0	450	4400	41.5 ± .56	0.635	25
6.0	720	7000	38.8 ± .18	1.30	25.5
6.0	900	8800	38.1 ± .24	1.45	22
6.0	1028	10000	37.2 ± .39	1.64	22
18.5	720	3700	42.6 ± .49	0.50	25.3
18.7	900	4100	41.9 ± .45	0.65	21.5
19.0	1200	6100	40.4 ± .23	0.98	21.2

Δ Values at t = 6.4°, η = 0.91

Δ Values for λ = 546 m	$G\eta$	$\Delta/G\eta c$
20.6	2900	0.0057
24.8	3450	.0057
30.5	4300	.0057
39.3	5700	.0055
45.4	6900	.0053
53.9	8600	.0050

From the measured value of $\eta\Theta/T$ the length of the molecule may be calculated if it is assumed for simplicity to be an ellipsoid of revolution. An approximate estimate of the axial ratio, derived for instance from viscosity measurements, must be employed in making this calculation, but the value of the derived length is very insensitive to the exact value. The length, l, assuming the molecule to be an elongated ellipsoid of revolution, is given by the equation

$$l^3 = 1.5\frac{kQ}{\pi}\left(\frac{\eta\Theta}{T}\right)^{-1} \qquad (1)$$

where k is Boltzmann's constant and $Q = -1 + 2\ln 2a/b$. For a/b we have taken the value 5.35 given by Oncley, Scatchard and Brown[9] and derived from viscosity and sedimentation data. This gives for γ-globulin a Q value of 3.74, so that the length ($2a$) in Å. becomes $l = 627(\eta\Theta/T)^{-1/3}$. Thus, the calculated length from the experiments in Table I is 224 Å., slightly below the value of 235 reported by Oncley, Scatchard and Brown.[9]

In Table II the results of these and other experiments on γ-globulin are summarized, and some of the data are reported also in Figs. 1, 2 and 3. It is immediately apparent that there is a marked downward trend in $\eta\Theta/T$ as the concen-

TABLE II

SUMMARY OF RESULTS ON γ-GLOBULIN (PREP. IIG1-L371)

Protein concn., C, g./100 cc.	Temp., °C.	Wt. % glycerol in solvent	Viscosity of solvent η	Range of $G\eta$ × 10^{-3}	$\eta\Theta/T$	$(\Delta/G\eta c)_0$
1.25	6.0– 6.3	76.0	0.93	2.85–10.0	22	0.0057
1.25	18.5–19.0	76.0	.49	3.7 – 6.1	22	...
2.5	6.1– 6.5	76.0	.94	2.9 – 9.1	14	.0071
2.5	19.0–19.8	76.0	.48	1.8 – 5.9	18	.0062
2.5[a]	6.2– 6.7	76.0	.90	2.8 – 8.4	19	.0070
2.5[a]	18.0–18.6	76.0	.49	2.3 – 6.2	21	.0055
3.3	6.1– 6.6	68.7	.42	0.6 – 4.0	9	.0100
3.3	19.5–19.9	68.7	.22	0.7 – 3.4	11	.0080
3.4	6.0– 6.5	60.5	.19	0.6 – 3.0	8	.0120
5.0	6.0	76.0	.91	1.1 – 6.9	7	.0100
5.0	18.0–19.2	76.0	.48	1.0 – 4.5	8	.0080

[a] Solution in acetate buffer, pH 3.93 before addition of glycerol. All other solutions studied at pH near 7.

(9) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 184 (1947).

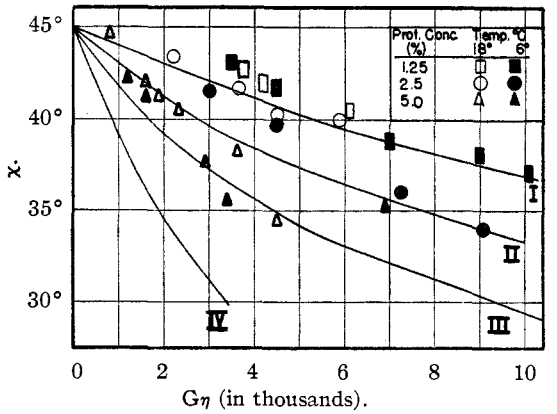

Fig. 1.—Value of χ as a function of $G\eta$ for solutions of human γ-globulin. Curves I, II, III, and IV are calculated as discussed in the text. Curve I is for the pure monomer, 235 × 44 Å. Curve IV is for the pure dimer, 470 × 44 Å. Curve II is for a mixture of 95% monomer and 5% dimer. Curve III is for a mixture of 85% monomer and 15% dimer. Points are experimental values derived under the conditions indicated in the figure.

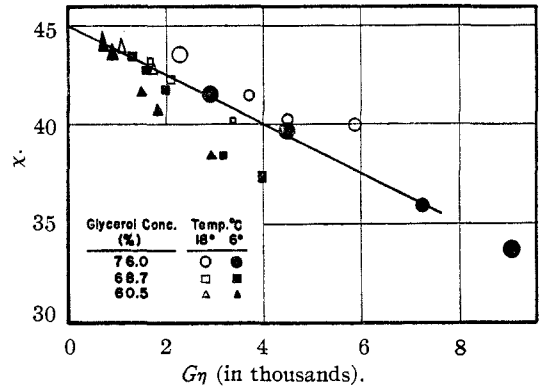

Fig. 2.—χ Values for human γ-globulin solutions as a function of $G\eta$ at various temperatures and glycerol concentrations. Protein concentration in all experiments indicated between 2.5 and 3.3 g./100 cc. Points are experimental values; the curve is calculated for a single molecule, for which $\eta\Theta/T = 13$.

tration of protein increases. This trend is similar to that already observed in the case of zein (concentration range 1 to 3%) and fibrinogen (concentration range 0.12 to 0.5%). Clearly it must be attributed to interactions between the protein molecules. As is to be expected, the influence of increasing concentration becomes apparent at relatively high dilutions in the case of fibrinogen solutions, where the molecules are very long. In the case of zein and γ-globulin, the concentration effects are marked only at concentrations considerably above 1%.

The effect of variation in glycerol concentration (Fig. 2) is rather less striking than that of variation in protein concentration. The observed χ values, at a given value of $G\eta$, deviate somewhat more from 45° in the solvents of low glycerol con-

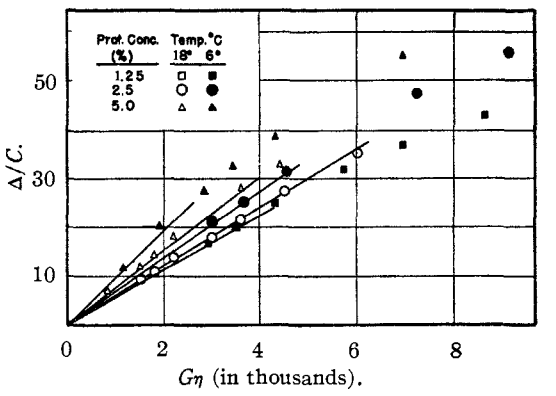

Fig. 3.—Double refraction of γ-globulin as a function of $G\eta$ for three different concentrations and two different temperatures. Ordinates are expressed as measured phase differences divided by concentration; these may be converted to double refraction values ($n_e - n_0$) by the conversion factor given in the footnote to Table IV.

tent and therefore of low viscosity. The effects, however, are relatively small and it is probably premature to attempt to interpret them at this time.

The effects of temperature are indicated in Figs. 1, 2, and 3 by plotting the results at low temperature using shaded symbols, and those at the higher temperature using open symbols. The χ values, plotted as functions of $G\eta$, appear very little affected by temperature. What small effects are discernible are in the direction that would be expected on the basis of decreased Brownian movement and increased protein–protein interaction at the lower temperature.

The specific double refraction values (Δ/c), when plotted as a function of $G\eta$ (see Fig. 3 and Table II) show a definite upward trend with increasing protein concentration and a slight downward trend with increasing temperature at constant concentration. Both these effects are in the direction that would be expected from the effects of concentration and temperature on protein–protein interaction.

In two experiments, indicated by asterisks in the first column of Table II, the pH of the γ-globulin solution was adjusted to 3.93 before glycerol was added. The results obtained in these runs were indistinguishable from others in the series, indicating that the size and shape of the γ-globulin molecules were unaltered in the more acid solution.

Calculations for a Two-Component System.—γ-Globulin preparations, of the type studied here, although electrophoretically homogeneous, show at least two major components in the ultracentrifuge. The sedimentation constant, s_{20}, is near 7.2 S for the major component (75–85% of the total) and approximately 10 S for the chief secondary component. Oncley, Scatchard and Brown,[9] who reported these data, tentatively interpreted them on the assumption that the $s = 10$ component

was a dimer, made up of two molecules of the $s = 7$ component associated end to end. The viscosity data were entirely compatible with this view. We have, therefore, examined the applicability of the same hypothesis to the double refraction of flow measurements.

For simplicity, we have assumed both monomer and dimer to be ellipsoids, the former being 235 Å. long, as assumed by Oncley, Scatchard and Brown,[9] and the latter 470 Å. long. The principal axis of the cross-section of both ellipsoids was taken as 44 Å. Such a model is certainly oversimplified, but the available evidence at this time scarcely warrants a more refined treatment. On calculating rotary diffusion constants for the ellipsoidal molecules, we find for the large molecule (component 1) that $\eta\Theta_1/T = 3.26$ and for the small molecule (component 2) that $\eta\Theta_2/T = 18.2$.

The fundamental equations for double refraction of flow in a polydisperse system have been given by Sadron[10] (see also Peterlin and Stuart[11]). The equations and figures in the papers of Peterlin and Stuart[11,12] give values of χ and of the relative double refraction, f, as a function of $\alpha = G/\Theta$, in a form which may be applied to each individual component with a given Θ value. In evaluating the double refraction given by the system composed of monomer and dimer, we have assumed that both components would give the same amount of double refraction at the same weight fraction in solution, if all the molecules in both solutions could be oriented with their major axes parallel.[13]

Values of χ were calculated for the two component system for two cases, assuming the weight fraction of dimer to be 0.15 in the first case and 0.05 in the second. The resulting curves for χ as a function of $G\eta$, for both these systems, are shown in Fig. 1, together with the corresponding curves for the monomer and dimer alone. Experimental data at several concentrations are indicated by the points in the figure. It is apparent that, at the two lowest concentrations studied, the experimental data approximate very closely to the calculated curve for the small component and show no evidence of the presence of the dimer. At the highest concentration of protein (5%), the data, for the most part, lie in the region between the two calculated curves for the polydispersed system. It is, therefore, possible to interpret the increased molecular interaction which is manifest at the higher protein concentrations, either as being due to an orienting influence exerted by each protein molecule on its neighbors in its field of flow, or to an actual association of some of the molecules into longer units.

(10) C. Sadron, *J. Phys. Radium*, [7] **9**, 381 (1938).

(11) A. Peterlin and H. A. Stuart, "Hand- und Jahrbuch der chemischen Physik," Band 8, Abschnitt IB, 1943, especially pages 88–91, inclusive.

(12) A. Peterlin, *Z. Physik*, **111**, 232 (1938); A. Peterlin and H. A. Stuart, *ibid.*, **112**, 1, 129 (1939).

(13) Details of the method of calculation for polydisperse systems will be given in a separate note by H. A. Scheraga and the present authors.

These results are obviously different from those of Oncley, Scatchard and Brown[9] who observed a considerable amount of the $s = 10$ component even at the lowest concentrations studied, and did not observe any great change in the ultracentrifuge diagram with change in protein concentration. However, their studies were carried out in aqueous solution, whereas ours were made in 60–76% glycerol. It is possible that the addition of glycerol causes a dissociation of the dimer molecules into the smaller units, and that under these conditions it is necessary to go to considerably higher protein concentrations before association occurs. The most striking conclusion from our own observations, however, is the remarkably good agreement at low protein concentrations between the experiments and the calculated values for a molecule approximately 235 Å. long, postulated as the main component by Oncley, Scatchard and Brown.[9]

We have based our calculation of a length of 220–230 Å. on the values obtained at the lowest protein concentration studied, and have not attempted to extrapolate the data to infinite dilution. If such an extrapolation were made on the basis of the data in Table II, using a linear plot of $\eta\Theta/T$ against concentration, we should obtain a still lower value for the length than that reported above, and the agreement with the other data would be less good. However, the basis for such an extrapolation is still uncertain and we have preferred, for the present, to use the experimental values at the lowest concentration studied.

Bovine Serum Albumin.—Only three experiments on serum albumin were carried out, and two of these gave rather fragmentary results, on account of the great technical difficulty of making observations in media of such extremely high viscosity. The results of the most extensive and satisfactory experiment are listed in Table III. In such experiments, the large amount of energy dissipation in the liquid during the maintenance of a velocity gradient leads to heating of the liquid to an extent which is difficult to compensate for by the circulation of water at constant temperature through the jacket of the outer cylinder. The dissipation of energy in the liquid per second is equal to $G^2\eta$ per unit volume.[14] Even more serious than the rise of temperature produced is the fact that thermal gradients are produced in the liquid, which can distort the path of the light beam and falsify the optical measurements. These difficulties have been discussed in detail by Björnståhl,[15] but we have not attempted to apply his quantitative calculations to our data, since the boundary conditions which Björnståhl assumed at the inner and outer cylinders do not correspond to the conditions existing in our apparatus. All these difficulties indicate that the results of the meas-

(14) See for instance, J. R. Robinson, *Proc. Roy. Soc.* (*London*), **A170**, 519 (1939), especially pages 540 ff.

(15) Y. Björnståhl, *Z. Physik*, **119**, 245 (1942).

urements on serum albumin solution are to be regarded with great caution.

TABLE III

DOUBLE REFRACTION OF FLOW OF BOVINE SERUM ALBUMIN (LOT 27-315)

Solvent: 88.45% glycerol; η at 5°, 4.5; at 21°, 1.76. Protein concentration: 4.48 g./100 cc.

t, °C.	Speed, R. P. M.	$G\eta$	χ	α	$\eta\Theta/T$	Apparent length, Å.
5.0	132	6250	41.5	0.74	30	190
4.5	212	10200	38.3	1.42	26	200
5.2	300	14000	35.6	1.92	26	200
5.0	360	17000	36.2	1.85	33	184
5.0	450	21300	34.2	2.3	33	184
20.6	450	8500	40.1	1.04	28	195
20.8	600	11300	40.0	1.06	36	179
21.0	720	13300	35.4	2.02	25	202
21.5	900	16100	34.4	2.22	25	202

Δ values at t = 4.5°			Δ values at t = 21.5°		
R. P. M.	Δ	$\Delta/G\eta c$	R. P. M.	Δ	$\Delta/G\eta c$
100	8.9°	0.00040	360	8.5°	0.00029
152	13.0	.00039	450	10.1	.00028
240	18.8	.00035	600	12.9	.00027
300	21.1	.00032	720	14.8	.00025
360	23.3	.00029	900	16.8	.00023
450	25.5	.00026			

Our values for $\eta\Theta/T$ are of the order of magnitude of 30 for serum albumin. For horse serum albumin (carbohydrate free) Oncley[16] has found from dielectric dispersion measurements two critical frequencies (0.44 and 2.1 megacycles, referred to solutions in water at 25°) corresponding to $\eta\Theta/T$ values of 41 and 195, respectively. The higher value would not be detectable in our apparatus, but the lower one is remarkably close to our own measurements, particularly in view of the great difference—200 to 500 fold—in the viscosities of the solvents employed in the two series of measurements. Recent dielectric dispersion studies on human and bovine serum albumin give rotary diffusion constants very close to those of horse serum albumin.[17] On the other hand, the molecular model assumed by Oncley, Scatchard and Brown[9]—namely, an elongated ellipsoid of revolution with a major axis of 150 Å. and a minor axis of 38 Å.—gives a calculated value of $\eta\Theta/T$ of 61. The length calculated from our own data is only about 30% greater than that of the model of Oncley, Scatchard and Brown. On the whole, in view of the difficult conditions under which our measurements were made, it may be concluded that the agreement is remarkably good, and our results may be taken as an approximate confirmation of those deduced[9] from ultracentrifuge, diffusion, viscosity and dielectric dispersion measurements.

(16) J. D. Ferry and J. L. Oncley, THIS JOURNAL, **60**, 1123 (1938); J. L. Oncley, *J. Phys. Chem.*, **44**, 1103 (1940).

(17) J. L. Oncley, personal communication.

Interpretation of Double Refraction Measurements (Phase Differences) for Protein Solutions.—Measurements of the magnitude of the double refraction in solutions of zein, fibrinogen, γ-globulin and serum albumin have been tabulated in this and preceding papers of this series. The data, when expressed as double refraction, $n_e - n_0$, divided by $G\eta c$, show a difference of more than 600-fold between fibrinogen and serum albumin. The values for γ-globulin and zein are roughly twenty times as large as those for albumin, and of the order of one-thirtieth of those for fibrinogen.

These differences, however, are largely a reflection of the very different degrees of orientation obtained, at the same $G\eta$ value, for protein molecules of various lengths. To compare the birefringence of the different proteins in solution, the ideal arrangement would be to obtain completely parallel orientation of the axes of all the protein molecules in each solution, and measure the birefringence under these conditions, which is obviously the maximum attainable for the given system. Such a direct measurement is of course impossible in practice, owing to the Brownian movement of the protein molecules, but the results that would be so obtained can be derived by calculation from the actual experimental data, employing the theoretical treatment of Peterlin and Stuart.[11,12] They have expressed the observed double refraction, $n_e - n_0$, at any given velocity gradient, as the product of an optical factor, $g_1 - g_2$, and an orientation factor $f(\alpha, a/b)$.

$$n_e - n_0 = \frac{2\pi\Phi}{n}(g_1 - g_2)\, f(\alpha, a/b) \qquad (2)$$

Here Φ is the volume fraction of protein in the system, n is the refractive index of the solvent, and a/b is the axial ratio of the ellipsoidal molecule.

The function f is the same orientation function previously mentioned in connection with the calculation for the two component system. At low velocity gradients (low α values) f reduces to the form

$$\lim_{\alpha \to 0} f = \frac{\alpha}{15}\frac{a^2 - b^2}{a^2 + b^2} \qquad (3)$$

When all the molecules of any given species are oriented with their axes parallel, f becomes equal to unity.[18] Hence, from the observed values of the two quantities, $(n_e - n_0)/G\eta c$ and $\eta\Theta/T = G\eta/\alpha T$, it is possible to evaluate the amount of double refraction for any of the proteins studied at the degree of orientation attained when $\alpha = 1$. One may then calculate the maximum double refraction, $n_e - n_0$, for a 1% solution at complete orientation. These values are given in the next to the last column of Table IV. The final column contains the optical anisotropy factor, $g_1 - g_2$, for each protein as calculated from equation 3.

It should be clearly recognized that even the

(18) See the definition of f given by Peterlin and Stuart, ref. 11 page 52, equation 51a.

TABLE IV

DOUBLE REFRACTION OF FIBRINOGEN, ZEIN, γ-GLOBULIN AND SERUM ALBUMIN IN SOLUTION

Protein	c in g./100 cc.	Solvent	$n^{20}D$	t, °C.	$\left(\frac{n_e - n_0}{G\eta c}\right)_0$ × 10^{10}	$\eta\Theta/T$	$\left(\frac{n_e - n_0}{c}\right)_{\alpha=1}$ × 10^8	$(n_e - n_0)^{1\%}_{max.}$	$g_1 - g_2$
Fibrinogen	0.32	Gl, 38.7%	1.3823	20.5	89	1.2	315	4.7×10^{-5}	0.00143
Fibrinogen	0.12	Gl, 53.6%	1.4035	18.0	72	1.0	210	3.2×10^{-5}	.00100
γ-Globulin	3.3	Gl, 68.7%	1.4259	19.7	3.4	11	110	1.8×10^{-5}	.00055
γ-Globulin	1.25	Gl, 76.0%	1.4368	6.0	2.4	22	150	2.4×10^{-5}	.00074
Zein	1.0	P. G.	1.4331	20.5	3.3	8	75	1.2×10^{-5}	.00036
Serum albumin	4.48	Gl, 88.5%	1.4561	20.5	0.12	30	11	1.9×10^{-6}	.00006

Gl, followed by a percentage figure, denotes a glycerol–water mixture containing the indicated percentage glycerol; P. G. is propylene glycol. The zein preparation was laboratory zein (Foster and Edsall). Values for refractive index ($n^{20}D$), for glycerol–water mixtures, from L. F. Hoyt, *Ind. and Eng. Chem.*, **26**, 329 (1934); for proyplene glycol, from A. G. Pukirev, *Trans. Inst. Pure Chem. Reagents* (Moscow), **15**, 45 (1937), as reported in *Chem. Abstracts*, **32**, 5378 (1938). Other symbols are explained in text, or previous tables. It should be noted that the double refraction values are given for $\lambda = 546$ mμ, although the available data for the refractive indices of the solvents are given for the sodium D line. The differences in refractive index for the two wave lengths, however, represent only second order effects in the phenomena considered here. For our apparatus (length of cylinder 7 cm.) and for light of wave length 546 mμ, $n_e - n_0$ is related to the observed phase difference Δ, by the equation: $n_e - n_0 = 4.24 \times 10^{-8}\ \Delta$.

values of $g_1 - g_2$ so calculated are not inherent characteristics of the protein molecules themselves. Actually $g_1 - g_2$ is a function of two terms, the intrinsic birefringence of the protein itself and the form birefringence which depends upon the differences in refractive index between the protein molecule and the solvent. The refractive index of most protein molecules for light of the wave lengths here considered is in the range 1.57 to 1.61.[19] For serum albumin, Armstrong, Budka, Morrison and Hasson[20] have calculated a value (nD) of 1.598, and for γ-globulin a value of 1.618. These values are for the anhydrous protein, however, and take no account of solvation. We have attempted to calculate the intrinsic birefringence of the proteins studied here, employing the value 1.60 as the mean refractive index of the protein, and using the values for the refractive index for the solvent given in Table IV.[21] Our calculations yielded negative values for the intrinsic birefringence of all the proteins studied; in other words, if these calculations are to be trusted, the axis of maximum polarizability in these proteins is perpendicular to the long axis of the molecules. We are not yet confident, however, that this conclusion is correct, since the effects of solvation may be important, and may vary from one solvent to another. To draw definite conclusions, it would be necessary to study these proteins in a variety of solvents with a much wider range of refractive index than we have yet employed.[22] It would appear, however, that fibrinogen is not only the most geometrically asymmetrical of all the molecules, but optically the most anisotropic, while serum albumin is the least. γ-Globulin, while less geometrically asymmetrical than zein, shows a higher degree of optic anisotropy. Both γ-globulin and zein were studied in solvents of very nearly the same refractive index; hence this conclusion should apply directly to the intrinsic birefringence of these molecules.

Discussion

The only previous study of double refraction of flow in serum albumin and globulin, of which we are aware, is by Sadron, Bonot and Mosimann.[23] The plasma fractions which they studied were obtained by ammonium sulfate fractionation, and the globulin fractions are not directly comparable to ours.[24] Their serum globulin preparations contained considerable lipid which, in glycerol–water mixtures, became detached from the protein and gave rise to birefringence of opposite sign from that produced by the protein. The γ-globulin preparations studied by us, on the other hand, were practically lipid-free, so that this complication did not arise. From the curves for extinction angle given by Sadron, Bonot and Mosimann[23] for their serum globulin, it is apparent that $\eta\Theta/T$ was of the order of unity but somewhat less; and hence only about one-twentieth of the value found for our γ-globulin. Their figure would correspond closely to that to be expected for a very elongated molecule of the order of 900 Å. in length, of the sort observed in sera of high antibody titer in several species of animals.[25]

(19) M. P. Putzeys and Mlle. J. Brosteaux, *Bull. soc. chim. biol.*, **18**, 1681 (1936). Values close to 1.57 are also obtained from measurements of the double refraction of protein fibers determined in media of varying refractive index; see for instance H. H. Weber, *Arch. ges. Physiol.*, **235**, 205 (1934–1935).

(20) S. H. Armstrong, Jr., M. J. E. Budka, K. C. Morrison and M. Hasson, THIS JOURNAL, **69**, 1747 (1947).

(21) The equation for resolving observed birefringence into form and intrinsic birefringences are given by Peterlin and Stuart, ref. 12, pages 13 and 135. There is a misprint in the formulas as given on page 13; the term L_1L_2 should read $L_1 - L_2$.

(22) The streaming birefringence of tobacco mosaic virus in glycerol–aniline–water mixtures was studied by M. A. Lauffer, *J. Phys. Chem.*, **42**, 935 (1938). The observed birefringence fell practically to zero in a solvent of refractive index near 1.57; hence this virus protein appears to possess little or no intrinsic birefringence.

(23) Ch. Sadron, A. Bonot and H. Mosimann, *J. Chim. Phys.*, **36**, 78 (1939).

(24) The species of animal from which their plasma was taken is not explicitly stated, but it would appear from the context that it was horse plasma.

(25) See for instance E. A. Kabat, *J. Immunol.*, **47**, 513 (1943). It is not necessary to assume that the lipoprotein in the globulin preparations of Sadron, *et al.*, was identical with the elongated component giving rise to double refraction of flow. It seems probable that the preparation contained several components and that the lipoprotein was a less elongated protein molecule than some of the others.

Sadron, *et al.*, could obtain no evidence of double refraction of flow in their crystallized serum albumin preparations. This is in qualitative accord with our own results, since the albumin preparation studied by us gave no detectible double refraction in 70% glycerol, and it was only when the concentration of glycerol approached 90% that a measurable degree of orientation was obtained. Some of the other albumin fractions studied in Sadron's work did show appreciable double refraction, with values of $(n_e - n_0) \times 10^{10}/G\eta c$ ranging from 0.2 to 1.1, as compared with 0.12 for our preparation. Corresponding values for Sadron's globulin fractions G_1 and G_2 were 3.1 and 5, respectively, very close to those for human γ-globulin as reported in Table IV. Thus, the double refraction values of Sadron's preparations are quite similar to ours, although the rotary diffusion constants differ considerably.

Oncley[16] has studied the dielectric dispersion of γ-pseudoglobulin from horse plasma. The two frequencies obtained by him correspond to $\eta\Theta/T$ values of 6 and 53, respectively. The former value is not far from that obtained by us for human γ-globulin in the highest concentrations studied; but, as already indicated, our values under these conditions are greatly influenced by association or molecular interactions, or both. It is probable that the horse γ-pseudoglobulin is a more elongated molecule than the main component of the human γ-globulin. The latter preparation contains a considerable amount of euglobulin, which precipitates at *p*H near 7 at very low ionic strength. Dielectric dispersion measurements on this preparation can be made, therefore, only on the pseudoglobulin component, and are not yet available for comparison.

In the previous study of fibrinogen,[4] we have given a detailed analysis of several possible molecular models, in light of all the available experimental evidence. In the case of albumin and γ-globulin, we have little to add to the discussion already given by Oncley, Scatchard and Brown.[9] The γ-globulin preparations certainly contain more than one component, and therefore a detailed analysis based on a model containing only a single component would hardly be profitable to carry out.[26] The results on serum albumin presented in Table III represent measurements made under extremely difficult conditions, on account of the high viscosity of the liquid and the small amount of double refraction observed. Under the circumstances, we consider it remarkable that the agreement between our data and those obtained from the ultracentrifuge, viscosity and diffusion measurements is as good as it is. Oncley, Scatchard and Brown[9] calculated a length for serum albumin of 150 Å., whereas our estimates would lead to a value of 190–200 Å. We believe that our value deserves far less weight than theirs, and present it simply as confirmatory evidence for the general consistency of our results and those obtained by other methods.

Summary

1. Double refraction of flow measurements have been made on purified human serum γ-globulin and crystalline bovine serum albumin, employing as solvents glycerol–water mixtures of high viscosity.

2. The measurements of γ-globulin lead to an estimated molecular length near 230 Å., in excellent agreement with the value deduced from ultracentrifuge, viscosity and diffusion measurements by Oncley, Scatchard and Brown.[9] The γ-globulin preparations, at least in dilute solution, behaved as if the protein molecules were uniform with respect to molecular length.

3. The measurements of serum albumin lead to an estimated length of 190–200 Å., but this value is considered less reliable than the figure of 150 Å. previously reported by Oncley, Scatchard and Brown.[9]

4. Critical comparison has been given of the amount of double refraction given by four different proteins under comparable conditions.

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(26) It should be remembered in this connection, that Oncley, Scatchard and Brown give dimensions for the *hydrated* protein molecule. Since their measurements were carried out in aqueous solution and ours in glycerol–water mixtures, the degree of solvation of the protein cannot be expected to be identical in these different media. Obviously some change in the frictional coefficient must occur when the protein is transferred from one solvent medium to the other. We have no basis at present for calculating what this change is likely to be, although it seems probable that it is small.