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PHYSICO-CHEMICAL STUDIES ON BOVINE γ-GLOBULIN

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SUMMARY

1. Fluorescence depolarisation measurements on bovine γ-globulin in neutral solution yield rotational relaxation times which are much lower than expected from the translational dynamic properties of the molecule. Only a small further reduction in the relaxation time is caused by the presence of 6 M urea.

2. With the simple dispersion, low λ_c (2130 Å) and $-b_0$ values obtained from optical-rotation measurements over a range of wavelengths, it is suggested that non-polar amino acid residues may be of importance in controlling internal folding within the core of the molecule but that a considerable measure of internal flexibility still remains outside this core.

3. The action of LiBr on bovine γ-globulin is complex. In addition to the small change in optical-rotatory properties at low LiBr concentrations, an opposite trend, probably involving denaturation, occurs at higher LiBr concentrations. Beyond 5 M LiBr, a considerable reduction in solubility is observed.

INTRODUCTION

The effects arising from the presence in protein molecules of loosely connected sub-units possessing some rotational freedom with respect to one another have been discussed by WEBER[1]. In the case of fumarase, MASSEY[2] and JOHNSON AND MASSEY[3] obtained evidence in support of such an occurrence from measurements of depolarisation of fluorescence and sedimentation velocity. More recently in a study of the seed globulin, legumin[4], the relaxation time computed from fluorescence depolarisation was found to be some six times smaller than that expected on the basis of the translational kinetic properties of the molecule. It was therefore suggested that the relaxation time was determined either by the rotation of sub-units, or by general flexibility within the molecule. A decision between these possibilities was not then possible.

Experiments carried out over a number of years in this laboratory have indicated that a similar situation exists for bovine γ-globulin. Thus OTTEWILL[5] working on horse γ-globulin, RICHARDS[6] and CHADWICK AND JOHNSON[7] working on bovine γ-globulin found, by the fluorescent depolarisation method, values of the rotational relaxation time in the range $4-13 \cdot 10^{-8}$ sec. On the other hand, the harmonic mean relaxation time at 20°, computed from the dielectric dispersion data of ONCLEY[8]

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for the single component relaxation times has the value $45 \cdot 10^{-8}$ sec and for bovine γ-globulin, the value calculated from translational kinetic measurements is $42 \cdot 10^{-8}$ sec (see later). This paper describes further fluorescence depolarisation studies on bovine-γ-globulin not only in media of neutral pH, but also in the presence of the reagents, urea and lithium bromide, which are thought to modify profoundly the internal structure of a protein.

To aid in the interpretation of results, parallel measurements of sedimentation velocity and of optical rotation over a range of wavelength have also been made.

The conclusion thus emerges that there is considerable general internal flexibility within the molecule of bovine-γ-globulin. It also becomes apparent that the action of LiBr on proteins is of considerable complexity and is not to be interpreted merely as the promotion of helical structures[9,10]. The suggestion of BIGELOW AND GESCHWIND that LiBr may cause denaturation over certain ranges of concentration is supported by observations reported here for γ-globulin.

MATERIALS AND METHODS

Bovine γ-globulin

The bovine γ-globulin used was an Armour preparation (Fraction II, Lot No. DK 2172).

Buffer solutions

Buffer salts were of analytical reagent or equivalent grade and for most experiments, phosphate–NaCl mixtures were used (pH 7.8). In such cases, an ionic strength of 0.05 was contributed by phosphate according to GREEN[11] and NaCl was added to give a total ionic strength of 0.1 or 0.2. Urea was a May & Baker product with purity in excess of 99.5 % and LiBr was a B.D.H. Laboratory reagent. LiBr concentrations were computed from refractive index measurements, assuming data tabulated in International Critical Tables (VII, p. 73).

The fluorescent dye and its reaction with the protein

1-Dimethyl-amino-naphthalene 5-sulphonyl chloride (naphthyl dye), prepared as described by JOHNSON AND RICHARDS[4], was conjugated to the protein for the fluorescence depolarisation and other measurements as described by WEBER[12]. To 10 volumes of the stirred protein solution in phosphate–NaCl buffer (pH 7.8), at $\mu = 0.2$, 1 volume of acetone was slowly added at $18 \pm 2^{\circ}$. Then an amount of naphthyl dye equal to 2 % of the weight of the dissolved protein was added in the minimum volume of acetone, stirring being continuously performed. After allowing the turbid reaction mixture to stand at 2–4° until clear, it was centrifuged and then dialysed at 2–4° against a larger volume of buffer. Remaining traces of free naphthyl dye were removed by passing the solution through a Dowex-2 ion-exchange column, and the absence of free dye was tested for by ascending chromatography[13]. The degree of labelling was determined from absorbancy measurements at 3300 Å, the absorption maximum of the naphthyl dye, corrected for the absorption due to the protein itself. The molar absorption coefficient was assumed to be $4.3 \cdot 10^{-4}$ cm³/mole.

Depolarization of fluorescence

Following the treatment of Perrin, WEBER[13] has shown that the fluorescence

depolarization behaviour of macromolecules behaving as rigid ellipsoids of revolution may be described by

$$\frac{1}{p} + \frac{1}{3} = \left(\frac{1}{p_0} + \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right) \qquad (1)$$

where p is the degree of polarization of the fluorescent light emerging from the solution at 90° to the incident, unpolarized light; p_0 is a constant, τ is the mean life-time of the excited state of the fluorescent conjugate and ρ is the harmonic mean of the relaxation times of the macromolecule for rotations about the different axes of the hypothetical ellipsoid at the temperature T and viscosity η of the solution. Since ρ is proportional to η/T, a plot of $1/p$ *versus* T/η is usually linear. From the intercept on the $1/p$ axis and the interpolated value, $1/p_1$, at any value T_1/η_1, the corresponding value $\rho_1/_{3\tau}$ is readily calculated. Knowing the value of τ from independent experiments, ρ_1 may be obtained. STEINER AND MCALISTER[14] have suggested a τ value for the naphthyl dye of $1.18 \cdot 10^{-8}$ sec which is to a good approximation independent of the protein and nature of the solvent over a wide pH range.

Since the relative viscosity of dilute salt and many other solutions varies little with temperature, for convenience $1/p$ has been plotted against T/η_0, where η_0 is the viscosity of water at temperature T. The relaxation times so obtained are corrected to the viscosity of water by dividing by the relative viscosity of the solvent at that temperature[4].

The apparatus used to excite the fluorescence and to measure its degree of polarisation has been described in detail[15]. To confirm that it was working satisfactorily, the relaxation time for bovine serum albumin (Armour), labelled with the naphthyl dye, in phosphate–NaCl buffer (pH 7.8) at $\mu = 0.2$ was determined from a plot of $1/p$ *versus* T/η. The plot was linear and yielded a ρ°_{20} value of $11.5 \cdot 10^{-8}$ sec in reasonable agreement with that ($12.1 \cdot 10^{-8}$ sec) of WEBER[12] when the same τ value is assumed and with other estimates from this laboratory[16].

Optical rotation

Optical rotation measurements were carried out at $20 \pm 1°$ on a Rudolph Photoelectrical Polarimeter equipped with an oscillating polarizer. A Xenon arc was used with a Beckman Monochromator to provide illumination over the wavelength range 3500–7000 Å. Values of the specific rotation for the sodium D line ($[\alpha]_D$) quoted here have been corrected to the refractive index of water at 20° ($n_{D,w}$) by the equation

$$[\alpha]^{20}_{D,w} = [\alpha]^{20}_{D,w} \times \frac{n^2_{D,w} + 2}{n^2_{D,w} + 2} \qquad (2)$$

where $n_{D,w}$ is the refractive index of the solvent. Except at the higher LiBr concentrations, protein solutions contained approx. 1 g protein/100 ml and were centrifuged at 20000 rev./min before use in the 1-dm polarimeter tubes provided with quartz end plates.

Sedimentation

A Spinco Model E Analytical instrument, equipped with diagonal schlieren optics and phase plate was used. Rotor temperature was read throughout the experiments.

RESULTS

Sedimentation measurements

Ultracentrifuge examination showed that solutions of γ-globulin at 14–18° in phosphate–NaCl buffers (pH 7.8) (Fig. 1a) contained one well-defined main boundary with 5–15% of more rapidly sedimenting material with $s_{20,w} \approx 10$ S. A similar sedimentation pattern was also obtained at 4°. On conjugating with the naphthyl dye to the extent shown in Table I, a small increase in the proportion of the 10-S material was noted (up to 20%) but sedimentation coefficients were not affected (Fig. 2). The least squares line for the main component of unconjugated bovine γ-globulin is given by

$$s_{20,w} \text{ (Svedberg units)} = 7.00\ (\pm 0.06) - 0.18\ (\pm 0.07)c \qquad (3)$$

where c is measured in g/100 ml. In addition to the above results, Fig. 2 contains sedimentation data for γ-globulin which was exposed for long periods (>5 h) to various concentrations of LiBr (0.9 M, 3 M, 6.2 M) before removal of LiBr by thorough dialysis against several large volumes of 0.15 M saline. It is to be noted that, for LiBr concentrations up to 3 M, little or no precipitation of protein occurred and that the corrected sedimentation constants for the recovered material in saline fall well upon the control regression line. The sedimentation pattern (Fig. 1b) also resembled that of the control protein, though the amount of 10-S material was somewhat

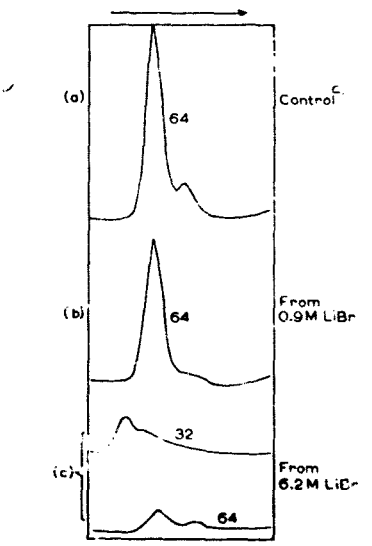

Fig. 1. Sedimentation paterns for bovine γ-globulin at 52640 rev./min at approx. 14°. (a) Control in phosphate–NaCl buffer at $\mu = 0.1$ (pH 7.8). Protein concentration 1.24 g/100 ml. (b) Recovered from 0.9 M LiBr in 0.154 M NaCl. Protein concentration 0.88 g/100 ml. (c) Recovered from 6.2 M LiBr in 0.154 M NaCl. Protein concentration 0.96 g/100 ml. Times in minutes given alongside each curve.

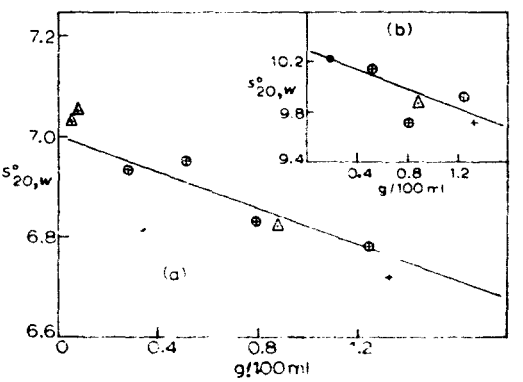

Fig. 2. Plots of corrected sedimentation coefficient ($s_{20,w}$) against total protein concentration for main (a) and 10-S (b) components. ⊖–⊖, Control γ-globulin; +–+, conjugated γ-globulin; △–△, γ-globulin recovered from 0.9 M LiBr; ▲–▲, conjugated γ-globulin recovered from 3 M LiBr; ●–●, γ-globulin recovered from 6.2 M LiBr.

variable. In this connection it was of interest that when the sedimentation was studied in 0.9 M LiBr, the proportion of 10-S material appeared much reduced. Although the sedimentation constants for the 10-S component were not as accurate as for the main species, a plot of its $s_{20,w}$ against total protein concentration (Fig. 2b) indicated that conjugation and exposure to high LiBr concentration did not modify appreciably its sedimentation behaviour.

TABLE I

THE EFFECT OF DEGREE OF LABELLING ON DEPOLARISATION PARAMETERS

Degree of labelling (moles/mole)	$p_0 (\pm 0.01)$	$\rho^\circ_{20} \times 10^8 (\pm 0.5)$
3.60	0.267	8.6
3.90	0.278	8.2
4.22	0.274	7.9
4.36	0.268	7.55
5.30	0.280	9.3
6.52	0.275	8.5

On the other hand during exposure to 6.2 M LiBr, approx. 65 % of the protein was lost by precipitation and the sedimentation pattern in 0.154 M saline of the remaining soluble material (Fig. 1c) was very different from the control. Approximately one sixth sedimented slightly more slowly than the control material at the same concentration (6.7 instead of 7.0 S), the remaining material being much more rapidly sedimenting but so polydisperse as to give rise to no well-defined boundary.

Depolarisation measurements

Fig. 3 shows a plot of $1/p$ *versus* T/η for a naphthyl conjugate of bovine γ-globulin in phosphate–NaCl buffer (pH 7.8) $\mu = 0.2$. The plot is linear within experimental error for the range 0°–50° and yields a p_0 value of 0.27 ± 0.01. Taking τ to be $1.18 \cdot 10^{-8}$ (see ref. 14), the relaxation time in water at 20°, ρ°_{20}, is calculated to be $8.6 \cdot 10^{-8}$ sec, with an estimated error of $\pm 0.5 \cdot 10^{-8}$.

Variation in the degree of labelling over a two-fold range (Table I) did not within experimental error modify the linearity of the $1/p$ *versus* T/η plots or the p_0 and ρ°_{20} values. However, with 0.154 M NaCl as solvent in place of the phosphate buffer, whilst the $1/p$ *versus* T/η plot remained linear (Fig. 6) and p_0 was unaltered, the relaxation time at 20° became $11.3 \cdot 10^{-8}$ sec. It seems clear that specific buffer solvent or pH effects on the relaxation time may occur, but in this work they were not explored further.

The possible occurrence of irreversible temperature effects (as for the seed globulin, legumin[4,15]) was investigated for γ-globulin as indicated in Fig. 4. After performing p measurements over the range 20° → 50°, the conjugate in phosphate–NaCl buffer was heated at 55° for 4 h and the measurements were then made on cooling from 50° → 20°. A final p series was taken with increasing temperature. Clearly the $1/p$ *versus* T/η plot is displaced upwards by the heating at 55°, giving $p_0 = 0.235$ and a ρ°_{20} value of $9.9 \cdot 10^{-8}$. On the other hand from the linearity over the main part of the plots and good agreement of the measurements (for most of the temperature interval) taken with ascending and descending temperature, it seems that exposure to temperatures below 50° has little or no effect.

The effect of urea

Varying amounts of solid urea were added to solutions of the naphthyl γ-globulin conjugate in the phosphate–NaCl buffer, which were then made up to standard volume and thermostatted at 20°. Even at 6 M urea, no significant change with time in the p value at 20° was observed on standing for 5 h. Fig. 5 shows $1/p$ *versus* T/η plots for several urea concentrations, and Table II contains p_0 data and corrected relaxation times, ρ°_{20}. Little or no change in the ρ°_{20} values with urea concentration occurs up to 2 M urea but a significant lowering is observed at 6 M. On the other hand, p_0 decreases throughout the range.

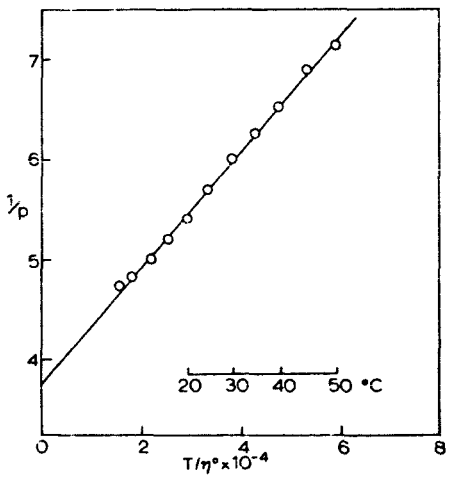

Fig. 3. Plot of $1/p$ *versus* T/η° for naphthyl conjugate of bovine γ-globulin in phosphate–NaCl buffer at $\mu = 0.2$ (pH 7.8).

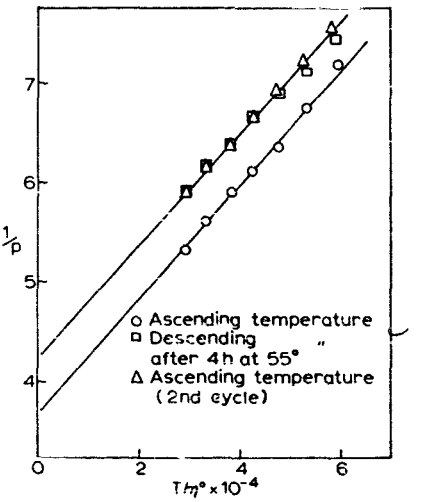

Fig. 4. Plots of $1/p$ *versus* T/η° for naphthyl bovine γ-globulin in phosphate–NaCl buffer during the following successive stages of heating and cooling: O—O, 20° → 50°; □—□, 50 → 20° after heating at 55° for 4 h; Δ—Δ, 20° → 50°.

TABLE II

THE EFFECT OF UREA AND LiBr ON DEPOLARISATION PARAMETERS

Reagent	*Molarity*	$p_0 (\pm 0.005)$	$\rho^\circ_{20} \times 10^8 (\pm 0.5)$*
Urea	0	0.283	9.0
	1	0.253	8.5
	2	0.250	8.45
	6	0.235	6.80
LiBr	0	0.278	11.3
	1	0.278	12.4
	3	0.278	13.7
	5	0.308	6.3

* Values of relaxation time have been corrected by dividing by the relative viscosity of the solvent.

The effect of lithium bromide

To avoid the precipitation occurring when LiBr is added to phosphate buffer systems, conjugation of the γ-globulin was performed in 0.154 M NaCl solution to

which NaOH had been added to give pH 9. 10 M LiBr solution in distilled water was added to give the desired LiBr concentration. As already mentioned, at LiBr concentrations greater than 3 M, an increasing proportion of protein was precipitated with increasing LiBr concentration, (about 65 % at 6.2 M LiBr), and some further precipitation occurred when the LiBr was removed by dialysis against [illegible] NaCl.

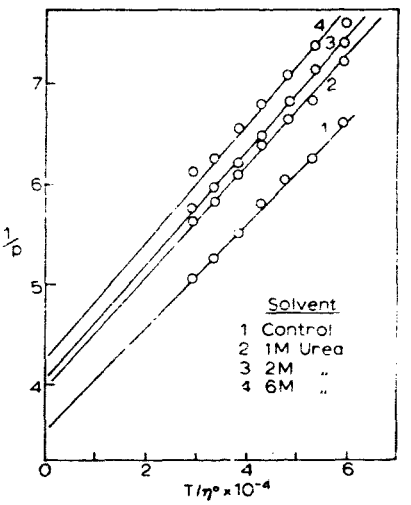

Fig. 5. Plots of $1/p$ *versus* T/η° for naphthyl bovine γ-globulin in phosphate–NaCl buffer containing various urea concentrations: 1, None; 2, 1 M; 3, 2 M; 4, 6 M.

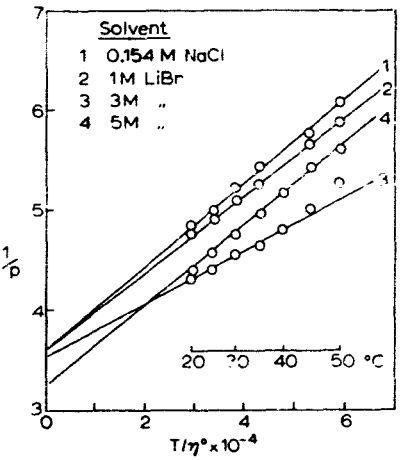

Fig. 6. Plots of $1/p$ *versus* T/η° for naphthyl bovine γ-globulin in 0.154 M NaCl solution containing various LiBr concentrations; 1, None; 2, 1 M; 3, 3 M; 4, 5 M.

At 20°, p values remained constant for 5 h with LiBr concentrations up to 5 M. Apart from the data at 3 M LiBr, the $1/p$ *versus* T/η plots (Fig. 6) are linear, and derived p_0 and ρ°_{20} values are given in Table II. As the LiBr concentration increases, ρ°_{20} increases significantly but above 3 M, lower values are obtained and, in 5 M LiBr, the relaxation time observed is very comparable with that for 6 M urea. However the latter results apply only to the fraction of γ-globulin remaining in solution.

Optical rotation measurements

Solid γ-globulin was dissolved in the appropriate LiBr solution, and after exhaustive dialysis against a large volume of the same LiBr solution, the protein concentration was determined in a Zeiss interferometer assuming a dn/dc of 0.00192 (n = refractive index, c = concentration in g/100 ml). Numerical data are collected in Table III and the variation of $[\alpha]$, the specific rotation with wavelength (λ) is shown (Fig. 7) as a plot of $[\alpha]\lambda^2$ *versus* $[\alpha]$ (see ref. 17).

At low concentrations of LiBr (< 3 M), $-[\alpha]_D^{20}$ decreases significantly with increasing LiBr concentration but at higher concentrations, an opposite trend of larger magnitude occurs. A similar parallel change in relaxation time with LiBr concentration has been described above. The good linear plots of $[\alpha]\lambda^2$ *versus* $[\alpha]$ for the differing LiBr concentration are in accordance with Drudes single term equation,

$$[\alpha] = \frac{k}{\lambda^2 - \lambda_c^2} \qquad (4)$$

and from the slopes, the λ_c values of Table III were obtained. With increasing concentration of LiBr, the plots of $[\alpha]\lambda^2$ *versus* $[\alpha]$ are at first displaced downward but this trend is more than reversed at 4 M LiBr. Similarly λ_c first increases but later decreases with increasing LiBr concentration.

In treating their results on β-lactoglobulin, TANFORD *et al.*[18] used MOFFITT AND YANG'S[19] equation,

$$[m^1] = \frac{3}{n^2+2}\frac{M_0}{100}[\alpha] = \frac{a_0\cdot\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\cdot\lambda_0^4}{(\lambda^2-\lambda_0^2)^2} \qquad (5)$$

where $[m^1]$ is the average rotation per peptide unit of molecular weight, M_0, in vacuo at wavelength λ; n is the refractive index of the solvent, a_0, b_0, and λ_0 being adjustable parameters. Using $\lambda_0 = 2120$ Å, b_0 values were calculated to be -70 (± 20) and were not affected by pH changes and denaturation. This low magnitude was taken to indicate the occurrence either of no α-helical regions in the protein or equal amounts of right and left hand helices. Applying the same type of analysis to the bovine γ-globulin results, b_0 values of even smaller magnitude ($0 > b_0 > -50$) were obtained and similar conclusions would seem to apply.

TABLE III

OPTICAL ROTATION DATA FOR BOVINE-γ-GLOBULIN IN LiBr SOLUTIONS AT 20°

Molarity of LiBr	$-[\alpha]_D^{20}$ ($\pm$ 0.5)	λ_c(Å)	$(-b_0)$ (Eqn. 5)
(0.154 NaCl) No LiBr	46.3	2130	20
1	42.0		
3	38.9	2300	30
4	65.15	2170	0
5	78.4		

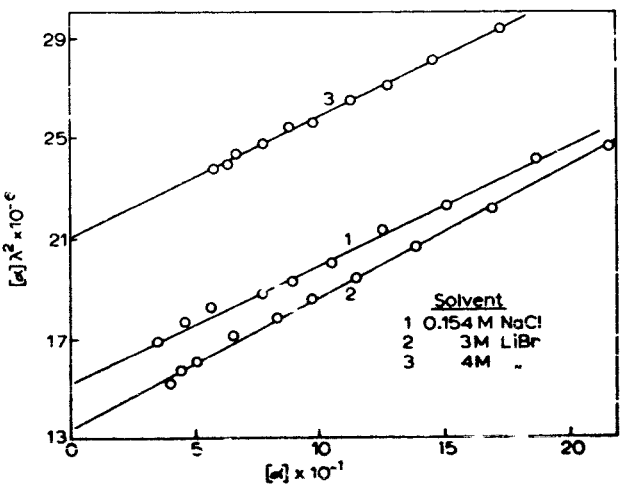

Fig. 7. Plots of $[\alpha]\lambda^2$ *versus* $[\alpha]$ for bovine γ-globulin at 20° in solutions containing 1, 0.154 M NaCl; 2, 3 M LiBr; 3, 4 M LiBr.

DISCUSSION

On the basis of the following physico-chemical data: $s_{20,w} = 7.0$ S; $D^{\circ}_{20} = 4.1 \cdot 10^{-7}$ (see ref. 20); $\bar{v} = 0.720$ (see ref. 21); $[\eta] = 0.067$*, (see ref. 22), we calculate the rotational relaxation time of the γ-globulin molecule in water at 20° to be approx. $42 \cdot 10^{-8}$ sec (see ref. 6). The low value of the relaxation time measured by the depolarisation method as compared with the above calculated value and its variability with type of neutral solvent and mild heating, suggest that even in the native state the bovine γ-globulin molecule possesses either considerable internal flexibility or possibly several sub-units capable of rotation with respect to one another.

Optical rotation measurements aid in further interpreting these results. Thus whilst the $[\alpha]_D$ value of −46.5° is normal for an undenatured protein molecule, the more decisive property, λ_c, has a value 2130 Å (in good agreement with other measurements[23]) which is usually accepted as indicative of a denatured or disordered structure. The linearity of the $[\alpha]\lambda^2$ *versus* $[\alpha]$ plots, or the adherence to a single term Drude equation is also in conformity with this picture.

JIRGENSONS[23] has drawn attention to the occurrence of a group of proteins with $[\alpha]_D$ values falling within the normal range for native globular proteins but whose λ_c values are more indicative of the denatured state. Several of these proteins possess various forms of biological activity and it would therefore be unlikely in general that this behaviour arises from the occurrence of a highly disordered internal structure. Rather it is to be expected that the folding of the polypeptide chain is determined by forces other than those (particularly hydrogen bonds) operating in the more typical cases. PERLMANN[24] has drawn attention to the possible role of non-polar side chains and proline in the case of pepsin, and TANFORD *et al.*[18], in a study of β-lactoglobulin, have suggested that the internal structure is determined largely by the hydrophobic interactions of the non-polar side chains. Similar considerations may well apply to bovine γ-globulin, but to conform with the measurements of rotational relaxation time, it seems necessary also to postulate a considerable degree of internal flexibility within the molecule. Some form of tight close-packing of hydrophobic side-chains in the core of the molecule with a more flexible, less ordered conformation outside is envisaged. The alternative explanation of the relaxation results in terms of discrete, tightly-knit sub-units capable of rotation with respect to one another seems unlikely, since this type of structure would be expected to give optical rotation behaviour of the more normal protein type. The occurrence of sub-units possessing internal flexibility is not, however, excluded. The rather small effect of 6 M urea on the relaxation time (reduction of 25 %) as compared with the case of the seed globulin, legumin[4,15] is also suggestive of an initial structure which is either partially disorganized or stabilized by means other than hydrogen bonds. The special biological role of γ-globulin may well be dependent upon the occurrence of flexibility within the molecule.

It is of some interest that fluorescence depolarisation measurements on conjugates of bovine γ-globulin with a pyrene derivative yielded the significantly higher relaxation time of approx. $30 \cdot 10^{-8}$ sec (see ref. 7). Though this also is significantly

* The $[\eta]$ value used is a mean value for human γ-globulin fractions. No value for bovine γ-globulin is available but the uncertainty involved does not detract from the conclusion drawn that the calculated relaxation time is much greater than that obtained from depolarisation studies.

smaller than the theoretical value calculated for the rotation of a rigid molecule with the translational properties given above, it does seem that the extent of the internal flexibility may be modified by the presence of adsorbed molecules.

If the native structure of the γ-globulin is indeed disordered, it might be expected that considerable changes in measured physico-chemical properties would occur in the presence of agents which promote the formation of hydrogen bonds*. The increase in rotational relaxation time and in λ_c, the fall in $-[\alpha]_D^{20}$ and the displacement of the $[\alpha]\lambda^2$ *versus* $[\alpha]$ plot with LiBr content at low concentrations, may indicate some tendency towards a more ordered and less flexible internal configuration. Such changes are similar in direction to those reported by HARRINGTON AND SCHELLMAN[9] for clupein and oxidized ribonuclease in LiBr solutions, but their extent is much more limited. Little if any change in b_0 occurs.

At higher LiBr concentrations, the trends in relaxation time, in λ_c, and $-[\alpha]_D$ with LiBr concentration are reversed and at 4–5 M LiBr, $[\alpha]_D$ and λ_c have values usually associated with the denatured state, with b_0 indistinguishable from zero. The overall variation of $[\alpha]_D$ with LiBr concentrations is similar to that for ribonuclease[9,10] up to approx. 5 M LiBr, but the final fall in $-[\alpha]_D$ for ribonuclease at higher LiBr concentrations could not be observed for bovine γ-globulin since a large proportion of the protein had precipitated at this stage. Since a "fractionation" of the γ-globulin system may well have occurred thus, the results at higher LiBr concentrations are strictly not comparable with those at lower concentration where the protein is completely soluble. These findings support the view of BIGELOW AND GESCHWIND[10] that LiBr has a denaturing effect on proteins at higher concentrations, which is to be considered alongside any other changes which may occur.

The mechanism by which LiBr causes denaturation is of considerable interest. Since γ-globulin contains a significant proportion of amino acid residues with non-polar side chains, the considerable solubility of the molecule under normal conditions probably depends on the efficient internal packing of such side chains away from the solvent. Whatever the mechanism by which LiBr acts upon a protein, there is general agreement that its action is to modify profoundly the structure and it seems likely that this involves disturbance of the internal packing of non polar side chains, exposing them to the polar solvent and thus causing insolubility. The fact that precipitation occurs over a range of LiBr concentrations is in conformity with the range of properties usually associated with γ-globulin. In particular, the material remaining in solution at the higher LiBr concentrations must possess a highly stable internal structure to resist the denaturation process, though it is of note that its $-[\alpha]_D$ value has increased significantly. On the other hand its λ_c, and b_0 values are unchanged from those of the native γ-globulin molecule and characteristic of a random type of structure.

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* When this work was substantially complete, strong evidence against such action by LiBr was reported[10,25].

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