NOTICE: When government or other drawings, specifications or other data are used for any purpose other than in connection with a definitely related government procurement operation, the U. S. Government thereby incurs no responsibility, nor any obligation whatsoever; and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use or sell any patented invention that may in any way be related thereto.
STRUCTURAL TRANSITIONS IN ANTIBODY AND NORMAL γ-GLOBULINS.
II. FLUORESCENCE POLARIZATION STUDIES

RESEARCH REPORT

Report No. 18
Structural Transitions in Antibody and Normal γ-Globulins. II. Fluorescence Polarization Studies

The effects of acid, alkaline, anionic and cationic detergents, urea and guanidine on the relaxation times of rabbit anti-thymoglobin antibody and bovine γ-globulin have been evaluated by means of the polarization of fluorescence technique. Both proteins were coupled to dimethylaminonaphthalene sulfonyl chloride. More profound structural modifications occurred in detergent and urea solutions than in acid or basic solutions. However, the effects of alkali persisted even after the globulins had been extensively unfolded by detergents or urea. In contrast to the independent action of alkali and the organic reagents, sodium dodecyl sulfate and urea showed interdependent effects. Although the polarization of both protein preparations approached zero in alkaline in the presence of sodium dodecyl sulfate and urea, they were regained after neutralization and removal of the reagent by dilution. It would appear that γ-globulins are capable of a continuous unfolding to a state essentially free of noncovalent bonds.

It has come to be generally recognized that the spatial conformation of most globular proteins is capable of varying degrees of distortion from that characteristic of the state loosely denoted as native. The extent of distortion consistent with a total recovery of the native conformation varies from protein to protein. Such structural transitions can be mediated by a variety of external parameters, including pH, temperature, detergents and high concentrations of organic solutes and solvents, as well as combinations of these.

The existing molecular information upon the γ-globulins indicates clearly that they are no exception to the above. At least two molecular transitions are known from earlier work to take place in particular zones of pH.

In acidic solutions alterations in optical rotation, viscosity and sedimentation have been reported. The viscosity appears to depend on the ionic strength in an unusual way. In addition, the molecular composition, as measured by ultracentrifugation, has been reported to vary with the concentration and (type) of salt present. In alkaline solutions, an increase in optical rotation and reduced viscosity has been reported for human γ-globulin.

It is the purpose of the present set of papers to examine in detail the molecular state of the γ-globulins by as many criteria as are available. In particular, the present paper will supplement the molecular-kinetic and optical rotatory studies of the companion paper with additional information based upon the altogether different technique of fluorescence polarization. Properly used, this method can provide more direct information as to the internal organization of a protein than do the classical molecular-kinetic methods, which depend primarily upon the over-all shape and extension of the molecular domain.

Theory

It is unnecessary to dwell at length upon the now-well-known basic theory of fluorescence

(1) B. Jirgensons, Arch. Biochem. Biophys., 46, 184 (1944), 1946, fig. 46, 184 (1944).  
sphere of the same molecular weight. 10 The latter quantity ($\rho_a$) is given by $3N/VRT$ where $V = \text{molecular volume}$, $R = \text{gas constant}$. Values of $\beta$ greater than unity reflect the presence of molecular asymmetry or hydration.4 Values of $\beta$ less than unity can only result from the presence of internal degrees of rotational freedom.11

**Experimental**

**Preparation of Conjugates.**—Rabbit and bovine $\gamma$-globulin were coupled with the fluorescent compound 1-dimethylaminoethylate-9-sulfonyle chloride (DNS) under weakly alkaline conditions (0.1 M NaHCO$_3$, pH 8). The dimethylaminoethyle chloride was added in the form of a suspension produced by adding 1 ml of a 1.5% solution of DNS to acetone to 4 ml of water. One ml of the suspension was added to 10 ml of a 1-2% solution of globulin at $0^\circ$.

After 2 hr. the solution was centrifuged to clarity at 10,000 $g$; it was then dialyzed 48 hr. versus several changes of 0.5 M KCl. The next step consisted of three consecutive precipitations from 50% saturated (NH$_4$)$_2$SO$_4$ (3°), each precipitation being followed by solution in 10 ml of 0.01 M KCl.

Finally the solution was dialyzed 24 hr. versus 0.01 M KCl. It was then centrifuged at 20,000 $g$ for 2 hr. in a Spinco model L preparative ultracentrifuge. The final solution was frozen and stored at $-10^\circ$ prior to use.

**Degree of Conjugation.**—The average number of DNS groups per molecule of $\gamma$-globulin was determined spectrophotometrically. The molar concentration of DNS units was computed from the absorbancy at 280 m$\mu$ of a 0.5-1.5% solution of conjugate $\rho_a$ at pH 7. A value of $4.5 \times 10^{-3}$ was used for the molar extinction coefficient.5 The concentration of protein was determined from the absorbancy at 280 m$\mu$ of a suitably diluted solution. A value of 13.1 was used as the absorbancy of a 1% solution at pH 7 of bovine $\gamma$-globulin.

The degrees of labeling of the conjugates discussed in this paper are listed in Table I.

**Table I**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>DNS groups per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG-AG-II</td>
<td>3.6</td>
</tr>
<tr>
<td>BG-III</td>
<td>1.3</td>
</tr>
<tr>
<td>BG-V</td>
<td>1.0</td>
</tr>
<tr>
<td>BG-VI</td>
<td>2.2</td>
</tr>
</tbody>
</table>

**Measurement of Polarization.**—The polarization of fluorescent light at an angle of $90^\circ$ to the incident beam was measured by an adaptation of a Phoenix light scattering photometer using the same techniques described in earlier publications.10,11 Unpolarized incident light was used, a Polaroid analyzer being inserted before the photomultiplier tube. The fluorescent radiation was freed from any stray, scattered or reflected light by a pair of complementary filters. The incident beam was intercepted by a Corning 5070 filter and the fluorescent beam by a Corning 3385 filter inserted before the entrance slit of the photocell.

The cuvette, a 1 cm. quartz Beckman cell polished on all four sides, was positioned in a larger square cell equipped with glass walls through which water from a constant temperature bath could be circulated. In this manner, the temperature could be controlled to within $\pm 0.3^\circ$ over a range from 7 to 60$^\circ$.

**Materials.**—Rabbit anti-calf-thyroglobulin antibodies (TG-Ab) were obtained by the procedure of Metzger and Edelhoch.12 When characterized by ultracentrifugation and electrophoresis, 96% of the protein moved as a single boundary with physical constants typical of the $\gamma$-globulins.

Bovine $\gamma$-globulin (BG) was obtained from Armour and Co. Electrophoresis in 0.10 M barbital buffer, pH 8.66, showed only a single, rapidly spreading boundary. By ultracentrifugation in the same buffer only a small amount was observed (~5%) of a faster sedimenting boundary (9.55), which is normally found in $\gamma$-globulin prepared by the usual cold ethanol fractionation procedure.

Both rabbit and bovine $\gamma$-globulin preparations were slightly turbid at several $pH$'s at low salt concentrations, indicating the presence of euoglobulins. These solutions became clear at higher salt concentrations. Most of the euoglobulins were presumably removed in the course of preparation of the conjugates by the clarification step in 0.01 M KCl. The weight fraction of material thereby removed was negligible. No visible turbidity was observed for the conjugates at any $pH$ in 0.01 M KCl.

Sodium dodecyl sulfate (SDS) was a purified preparation donated by R. I. du Pont de Nemours and Co. Trimethyl-dodecylammonium chloride (TDAC) was obtained from Armour and Co. Salts were reagent grades. Urea was recrystallized from alcohol. All solutions were prepared with water distilled from an all glass apparatus.

A Leeds and Northrup pH meter equipped with Miniature external electrodes was used to measure $pH$. The instrument was calibrated with standard buffers at 4.00, 6.86 and 10.00.

**Computation of Relaxation Times.**—Values of $P_a$ were obtained by extrapolation of $p + \frac{1}{3}$ as a function of $T = \frac{2}{3}$. Equation 1 was then used to compute $\rho_aP_a$. A value of $\sigma$ equal to $1.3 \times 10^{-4}$, as found for other DNS conjugates,10 was used in calculation of $\rho_aP_a$, except in the case of BG where a value of $0.80 \times 10^{-4}$ was used.12

The extrapolation of $p + \frac{1}{3}$ was essentially linear at low temperature ($T < 30^\circ$) for the cases for which $\rho_a$ are cited. The values of $P_a$ varied to a minor degree with the conjugate and the external conditions. Thus for a single preparation of lambda rabbit antibody (TG-Ab-II) $P_a$ varied between 0.24 and 0.26. There was no very obvious drop in $P_a$ under conditions of maximum loss of rigidity. Indeed the largest value recorded, 0.26, was for the set of conditions (8 M urea, pH 12.4) for which $\rho_a(=\rho_aP_a)$ attained its minimum value.

However, in view of the enhanced uncertainty of extrapolation arising from the very high slope of $p + \frac{1}{3}$ as a function of $T$, and that prevailing at neutral $pH$ in the absence of denaturant (0.24) is probably not in excess of experimental error. The value of $P_a$ for the bovine globulin conjugate (BG-III) likewise ranged between 0.24 and 0.26. The unsystematic nature of the variation in $P_a$ arouses the suspicion that the differences are not outside of experimental uncertainty.

However, $\gamma$-globulin conjugates have been prepared for which $P_a$ attained a value as low as 0.19. The variation with conditions was again small. The similarity of the values of $\rho_a$ computed in this case to those cited for comparable conditions in Table II raises the possibility that the low value of $P_a$ may simply result from contamination with uncoupled dye.

Parenthetically, it may be mentioned that the dependence of $\rho_a$ upon external conditions to be discussed in the following sections cannot be accounted for by the acquisition of three rotation by the fluorescent groups alone, while the protein itself retains its initial rigidity. As has been pointed out by Weber,13 such behavior should diminish $P_a$ but leave $\rho_a$ unchanged—the very opposite of what is observed.

It must, however, be conceded that the values of $\rho_a$ computed under conditions of partial loss of internal rigidity represent rather poorly defined averages difficult to relate quantitatively to the structural parameters of the molecule. This by no means vitiates the usefulness of this quantity as a sensitive index of molecular rigidity.

The values of $\rho$ cited in Table II were computed using a value for the molecular weight of 1.6 $\times$ 10$^4$. This gave a value of $\rho_aP_a(=\rho_aP_a)$ equal to $1.3 \times 10^{-4}$. It is not intended to claim the highest precision for the values of $\rho_aP_a$ listed in Table II.

**Results**

**Relaxation Time of Native Protein.**—At an ionic strength greater than 0.1, data obtained by polarization of fluorescent light at the molecular configuration of $\gamma$-globulin, both immune and normal, does not change significantly between
June 5, 1962

**FLUORESCENCE**

sac., ular-kinetic behavior. Two globulins are of similar magnitude at pH 12 and essentially constant between pH 4 and 9. Outside very slow secondary stage is qualitatively in accord with pH occurs (Fig. 1). An analysis of the tension that the pH zone in which irreversibility becomes important corresponds closely to that of the loss of native 'globulin. This is not important for rabbit antibody and its degree of recovery upon back-titration become time dependent at pH 12. Vertical lines indicate experimental error.

The forward alkaline branch of the pH-polarization curve, which is similar for the two globulins, shows no important dependence upon ionic strength. The polarization profile is not completely in register with the observed variations of viscosity and sedimentation coefficient, as changes in polarization begin to be appreciable at distinctly lower pH's than in the latter cases.13

Immediate back-titration of the rabbit antibody from pH 12 to neutrality resulted in the recovery of most of the initial polarization (Fig. 1). However, the forward curve was not exactly retraced. It must be emphasized that the data of Fig. 1 represent the polarizations measured immediately (within 2 minutes) after titration of the solution to the indicated pH. In view of the time effects to be discussed later and the finite time required for measurement, it is difficult to make a quantitative statement as to the degree of reversibility from pH 12.14

Both the magnitude of the change in polarization for the rabbit antibody and its degree of recovery upon back-titration become time dependent at pH's alkaline to about pH 11.4 (Fig. 2). Above pH 12 the degree of reversibility declines very rapidly upon standing. It is of interest that the pH zone in which irreversibility becomes important corresponds closely to that of the loss of solubility and increase in levorotatory.14 Moreover the instantaneous initial drop in P and then the very slow secondary stage is qualitatively in accord with the kinetics observed by solubility and rota-

**TABLE II**

**ROTATIONAL RELAXATION TIMES OF RABBIT ANTIBODY** AND BOVINE y-GLOBULIN

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Solvent</th>
<th>pH</th>
<th>$\tau_0$</th>
<th>$\tau_0^f$</th>
<th>$\tau_0^b$</th>
<th>$\tau_0^f$ $\times 10^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG-Ab</td>
<td>0.1 M KCl</td>
<td>6.0</td>
<td>&gt;11</td>
<td>&gt;15</td>
<td>2.5</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>BC</td>
<td>0.1 M KCl</td>
<td>6.0</td>
<td>&gt;11</td>
<td>&gt;15</td>
<td>2.5</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>BG</td>
<td>0.1 M KCl</td>
<td>6.0</td>
<td>&gt;11</td>
<td>&gt;15</td>
<td>2.5</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>TG-Ab</td>
<td>0.2 M KNO₃</td>
<td>8.5</td>
<td>&gt;12</td>
<td>&gt;16</td>
<td>1.5</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>BG</td>
<td>0.2 M KNO₃</td>
<td>8.5</td>
<td>&gt;12</td>
<td>&gt;16</td>
<td>1.5</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>TG-Ab</td>
<td>H₂O</td>
<td>1.8</td>
<td>9.7</td>
<td>12.6</td>
<td>0.95</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>BG</td>
<td>H₂O</td>
<td>2.3</td>
<td>11.4c</td>
<td>14.8</td>
<td>1.1</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>TG-Ab</td>
<td>0.1 M KCl</td>
<td>11.9</td>
<td>4.5</td>
<td>5.8</td>
<td>0.45</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>BG</td>
<td>0.1 M KCl</td>
<td>12.1</td>
<td>4.8</td>
<td>6.2</td>
<td>0.38</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>TB-Ab</td>
<td>0.03 M TDAC</td>
<td>5.7</td>
<td>3.5</td>
<td>4.5</td>
<td>0.35</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>BG</td>
<td>0.03 M TDAC</td>
<td>5.8</td>
<td>2.6</td>
<td>3.4</td>
<td>0.26</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>TG-Ab</td>
<td>0.03 M TDAC</td>
<td>12.0</td>
<td>1.5</td>
<td>1.9</td>
<td>0.15</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>TG-Ab</td>
<td>0.012 M SDS</td>
<td>7.4</td>
<td>3.4</td>
<td>4.4</td>
<td>0.34</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>TG-Ab</td>
<td>0.02 M SDS</td>
<td>12.0</td>
<td>2.0</td>
<td>2.6</td>
<td>0.20</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>BG</td>
<td>0.012 M SDS</td>
<td>6.7</td>
<td>2.1</td>
<td>2.7</td>
<td>0.21</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>TG-Ab</td>
<td>0.03 M SDS</td>
<td>12.1</td>
<td>1.7</td>
<td>2.3</td>
<td>0.18</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>TG-Ab</td>
<td>8.0 M urea</td>
<td>7.0</td>
<td>4.5</td>
<td>5.7</td>
<td>0.45</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>BC</td>
<td>8.0 M urea</td>
<td>7.0</td>
<td>4.5</td>
<td>5.7</td>
<td>0.45</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>BC</td>
<td>0.1 M KNO₃</td>
<td>12.0</td>
<td>2.0</td>
<td>2.6</td>
<td>0.20</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>TG-Ab</td>
<td>8.3 M urea</td>
<td>12.4</td>
<td>4.0</td>
<td>5.5</td>
<td>0.40</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>BG</td>
<td>8.3 M urea</td>
<td>8.6</td>
<td>2.1</td>
<td>2.7</td>
<td>0.21</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
<tr>
<td>BG</td>
<td>8.0 M urea</td>
<td>12.5</td>
<td>0.6</td>
<td>0.7</td>
<td>0.06</td>
<td>1.2 $\times 10^{-3}$</td>
</tr>
</tbody>
</table>

* All the data for the rabbit antibody were obtained on TG-Ab-II. * All the data for the bovine γ-globulin were obtained on BG-III. Aggregation probably present.

pH 4 and 9. It is under these conditions that the 'native' molecule may be characterized. Some variation in the measured value of $\rho_0$ at neutral pH has been observed for γ-globulin conjugates of different degrees of labelling. In particular, bovine γ-globulin conjugates with apparent values of $\rho_0$ as low as 1.2 $\times 10^{-3}$ have occasionally been obtained. This may well result from a minor degree of denaturation in the course of preparation or from preferential labelling of a flexible region of the molecule. Those conjugates obtained for each species which had the largest values of $\rho_0$ at neutral pH were selected for detailed study as likely to be more representative of the intact molecule.

In view of this variation and the low precision of measurements of $\rho_0$ for proteins of relaxation time greater than 10⁻², it is not possible to assign an accurate figure for the absolute value of this parameter for native γ-globulin. This is not important for the purposes of the present paper, which is concerned primarily with changes in $\rho_0$. There is a definite possibility that the native molecule may not be completely rigid.

**Effect of Alkaline pH**—If the protein concentration and ionic strength are such that aggregation in the isoelectric region is avoided, the polarization of conjugates of γ-globulins of either species is essentially constant between pH 4 and 9. Outside of these limits a continuous decline in polarization with pH occurs (Fig. 1). An analysis of the temperature dependence at pH 12 reveals that the apparent value of $\rho_0$ is considerably reduced from its low polarization is time-dependent. It is of interest that the magnitude of the change in polarization for the rabbit antibody (TG-Ab-II) as a function of pH at 25°. The concentration is 0.26 g/l. Readings were taken immediately after attainment of the indicated pH. Vertical lines indicate experimental error.

The data for proteins of relaxation time are such that aggregation becomes important corresponds closely to that of the loss of native γ-globulin. This is not important for rabbit antibody and its degree of recovery upon back-titration become time dependent at pH's alkaline to about pH 11.4 (Fig. 2). Above pH 12 the degree of reversibility declines very rapidly upon standing. It is of interest that the pH zone in which irreversibility becomes important corresponds closely to that of the loss of solubility and increase in levorotatory. Moreover the instantaneous initial drop in P and then the very slow secondary stage is qualitatively in accord with the kinetics observed by solubility and rota-

---


(14) It is natural to raise the question of the significance of the relaxation times cited for rabbit globulin at alkaline pH's in Table II, since polarization is time-dependent under these conditions and the measurements at a series of temperatures require a finite time. In practice, after about 30 minutes at the indicated pH, the solution was chilled rapidly to 7-8° and then measured at a series of increasing temperatures between 1° and 30°. This required 20-30 minutes. Under these conditions changes in polarization during the run (as measured by rebinding the solution to 7°) were minimal (<2%). This is probably a consequence of the lowered rate of change at temperature below 25°.
In the case of the bovine \( \gamma \)-globulin, a wide deviation between the forward and reverse branches was noted (Fig. 3). The reverse branch was displaced strongly upward and undoubtedly reflects the presence of aggregation. This has been verified by direct ultracentrifugal examination of the reversed material. Sedimentation patterns of bovine globulin at pH 9.5 obtained immediately after an alkaline cycle showed the presence of a high proportion of rapidly sedimenting species. Aggregation occurs in the case of the rabbit globulin as well, but to a much less important extent.

The values of \( \beta \) computed for both globulins at pH's in the vicinity of 12 are considerably reduced from unity. Almost certainly this reflects the introduction of internal degrees of rotational freedom accompanying an alkaline structural transition to a looser and less compact molecular form than that prevailing at neutral pH. This model is entirely in harmony with the molecular-kinetic results and does not conflict with the observed changes in optical rotation.

**Effect of Acid pH.**—Although the exposure of \( \gamma \)-globulins of either species to pH's below 4 results in losses in internal rigidity which resemble those occurring in alkali, there appear to be major differences in molecular behavior produced by the two procedures. Beginning at pH 4 a continuous decline in polarization occurs, which, as seen in Fig. 1, attains its limiting value at pH 2, in the case of the rabbit antibody. The change is much less than that produced by alkali. Cann and Phelps have reported from ultracentrifugal analysis of bovine \( \gamma \)-globulin solutions that a molecular conformational change occurs between pH 4.2 and pH 3.5 which increases the frictional ratio of the molecule.

Ionic strength appears to have a somewhat greater influence upon the acid pH-profile of polarization than is the case in alkali (Fig. 1). While the drop in polarization persists at concentrations of KCl up to 0.10 and the initial phase of the pH-profile of polarization for the rabbit antibody appears to show no major dependence upon electrolyte concentration, the limiting value of the polarization varies considerably with ionic strength. About 75% of the fall in polarization that occurs with rabbit antibody between pH 5 and pH 1.9 was recovered when the KCl concentration was increased from 0 to 0.25 M at pH 1.9. It is of interest to note that Cann and Phelps have reported that a considerable increase in the sedimentation coefficient of the principal component occurs in bovine y-globulin as the salt concentration was increased from 0.02 to 0.2 NaCl, which they attributed to structural changes in the molecule.

From the analysis of the dependence of polarization on the viscosity of the medium, it is reported in Table II that the apparent relaxation time at pH 2 in the absence of electrolyte is less than that at neutral pH. In 0.1 M KCl at pH 2 a major increase in \( \rho_0 \) occurs. Cann and Phelps have shown that bovine \( \gamma \)-globulin also tends to aggregate as the salt concentration is increased at pH 3.1. The degree of masking of internal disorganization by aggregation phenomena cannot be readily evaluated.

Figure 3 depicts the effect of pH on the polarization of bovine \( \gamma \)-globulin. Data are presented also on the reversal of pH from both pH 2 and 12. There can be little doubt that the molecular events occurring in the acid are severely blurred by the presence of an important degree of aggregation in the case of the bovine globulin. Thus the acid branch of the pH profile of polarization for the bovine protein passes through a definite minimum on acidification even in the absence of added electrolyte (Fig. 3). The reverse curve is considerably elevated and the final polarization attained is larger than the initial. The presence of extensive aggregation limits the value of any extended discussion of the behavior of the bovine protein in acid. However, the minor fall of \( \rho_0 \) observed at pH 2.3 probably may be attributed to a transition similar to that occurring in the case of the rabbit protein.
June 5, 1962  FLUORESCENCE POLARIZATION STUDIES OF \( \gamma \)-GLOBULINS

**Fig. 4.**—SDS profile of polarization at pH 6.5 and at pH 12.0 for rabbit antibody (TG-Ab-II) in 0.02 \( M \) NaCl, 24\(^\circ\). The concentration is 0.26 g./l. 0, pH 6.5, forward, increasing SDS; \( \bullet \), pH 6.5, reverse, solvent dilution; \( \Delta \), pH 6.5, reverse, isoprotein dilution; W, pH 6.5, reverse after alkaline cycle to pH 12; O, pH 12.0, forward, increasing SDS; \( \oplus \), pH 12.0, reverse, solvent dilution.

**Effect of Detergent.**—The addition of the anionic detergent, sodium dodecyl sulfate (SDS) to a 0.02 \( M \) NaCl solution of antibody at neutral pH (6.5) results, after an initial lag, in a progressive drop in polarization which approaches its limiting extent at a detergent concentration close to 0.02 \( M \) (Fig. 4). Examination of the temperature dependence of polarization reveals that the apparent relaxation time in 0.02 \( M \) SDS is only about \( \frac{1}{4} \) its value for the intact molecule (Table II). A subsequent reduction in SDS concentration by dilution at neutral pH resulted in an ultimate recovery of a value of the polarization which was close to the initial. No important degree of hysteresis occurred between the forward and reverse branches (Fig. 4) at neutral pH. The dilution out of detergent was carried out both at constant protein:SDS ratio (dilution with solvent) and at constant total protein concentration (dilution with unlabeled rabbit \( \gamma \)-globulin solution). No major difference was observed between the two reverse curves (Fig. 4). Thus, the loss in rigidity resulting from the action of SDS at neutral pH is largely reversible. This, of course, does not necessarily imply a complete reformation of the initial fine structure.

In the presence of 0.0044 \( M \) SDS, an increase in pH from 6.5 to 12.0 resulted in a significant drop in polarization of the antibody (see Fig. 5). In elaboration of this pH-profile of polarization, it is seen from Table II that the relaxation time has decreased at pH 11.9, reverse; a fact difficult to reconcile with its value of neutrality. When the pH of the antibody solution was brought to 11.9 and titrated with SDS at constant pH, the same final value of the polarization was attained as in the reverse procedure. Back-titration to neutrality from 11.9 in SDS resulted in a partial recovery in polarization. However when the neutralized solution was diluted with solvent a high degree of recovery was ultimately observed (Fig. 4).

The changes of polarization induced by SDS appeared to attain completion rapidly and no important degree of time dependence was observed at either neutral or alkaline pH for the rabbit antibody.

When SDS was added to rabbit antibody at pH 12.0 and subsequently diluted out at the same pH, only a minor divergence occurred between the forward and reverse branches (Fig. 4).

The cationic detergent TDAC produces an effect upon the antibody molecule at pH 5.5 which is qualitatively analogous to that of SDS, except that a higher level of TDAC is required to yield an equivalent drop in polarization. In excess TDAC the relaxation time is similar in magnitude to its value at high concentrations of SDS (Table II). Below 0.007 \( M \) TDAC forms a turbid suspension with antibody, presumably by charge neutralization. This suspension redissolves at higher TDAC levels to yield a clear solution.

The polarization of the conjugated antibody shows no further change on reducing the pH from 5.5 to 2.5 in 0.024 \( M \) TDAC. However, as shown in Fig. 5 a very pronounced drop is observed between pH 9.5 and 12.0. Measurements of temperature dependence reveal a relaxation time greatly reduced from its value at neutral pH in TDAC (Table II).

It is of considerable interest to note that the relaxation time of antibody at pH 12 in excess TDAC is so low (\( P = 0.02 \) at 25\(^\circ\)) as to be difficult to measure with precision. The molecular conformation cannot differ greatly from a random coil. The attainment of such low values of polarization in a large protein molecule is of major interest since it implies that the higher values...
Fig. 8.—Urea profile of polarization for rabbit antibody (TG-Ab-II) at 26°. The concentration is 0.26 g/l. 0, forward, pH 8.1; 0, reverse, pH 8.1; A, reverse, pH 7.6, after an alkaline cytie to pH 12.2. The data are uncorrected for the increase in viscosity that occurs with urea concentration.

The influence of SDS in reducing the polarization of bovine γ-globulin is qualitatively in accord with the behavior of the rabbit antibody (Fig. 6). However, the degree of hysteresis in the reversal was greater and the extent of recovery less complete. This was true both for dilution with solvent and with unlabeled bovine γ-globulin at the same concentration. Completely comparable results were found when the same experiment was performed at pH 7.1 and at pH 9.5.

The influence of pH on the polarization of bovine γ-globulin in excess TDAC is seen in Fig. 7 to be basically similar to that on the rabbit antibody. However, there appears to be some divergence in behavior when solutions were made alkaline in excess TDAC. Thus the polarization of the bovine globulin shows no major change in 0.03 M TDAC between 5.5 and 11.3. However at pH 12.0 an immediate fall was observed which was followed by a rapid decline with time (Fig. 7). The effect of pH on bovine γ-globulin in 0.013 M SDS was quite different from the case of TDAC and resembled the behavior of antibody in SDS. The polarization of the bovine globulin in 0.013 M SDS fell practically linearly from 0.114 to 0.092 between pH 6.6 and pH 12.0. No time effects were apparent in these latter measurements.

The polarization of γ-globulin in 5 × 10⁻² M SDS (P = 0.136) was almost invariant to ionic strength. A slight (8%) decrease in polarization occurred between 0 and 0.2 M NaCl at pH 7. This, of course, is in vivid contrast to the behavior of the molecular-kinetic parameters and provides an illustration of the difference between those criteria which depend solely upon the molecular volume and porosity, and polarization, which depends upon the rigidity. Similar observations have been reported for the behavior of thyroglobulin in sodium decyl sulfate as the ionic strength was increased.¹⁹

References

Effect of Urea and Guanidine. — From the results discussed in the accompanying paper, it is apparent that high concentrations of urea produce both an inflation of the molecular domain, as judged by the molecular-kinetic measurements, and a transition in the internal structure, as reflected by a definite alteration in optical rotation. The latter property, in particular, appears to undergo a significant change at concentrations of urea somewhat above 6.0 M in the case of bovine $\gamma$-globulin. The rotatory properties exhibit a major degree of recovery upon removal of urea through a marked hysteresis is evident. In harmony with these observations, the addition of urea to $\gamma$-globulins of either species at neutral pH produces a drop in polarization, the steepness of whose variation increases significantly at levels of urea in the range $4-6$ M, as shown in Fig. 8. The reduction of urea concentration by dilution with solvent results in essentially complete recovery of the initial polarization. Little or no hysteresis is apparent in the reverse curves for globulins of either species.

Analysis of the temperature dependence of polarization in 8.5 M urea at neutral pH reveals an almost six-fold reduction in the value of $p$, for the bovine globulin. The effect is slightly less profound in the case of the rabbit antibody.

That the residual internal structure has not been brought to an irreducible minimum under these conditions is indicated by the further changes occurring at alkaline pH. Thus, in 9 M urea a continuous and dramatic decrease in polarization of the antibody occurs at pH's alkaline to 10 as seen in Fig. 9. The unfolding that occurs at pH 12.35 is incompletely reversible on neutralization since a rather small, but definite, hysteresis appears between the forward and reverse branches (Fig. 9). Almost identical results were obtained with bovine $\gamma$-globulin under similar experimental conditions. It should be noted that these pH-polarization curves are quite similar in shape to that reported above in aqueous media.

The apparent relaxation time at pH 12.1 is so low as to preclude the existence of an important degree of organized structure under these conditions. It is likely that the molecular conformation is essentially that of a random coil. As at neutral pH values, there is a quantitative difference between globulins of the two species (Table II).

Despite the almost totally unorganized character of $\gamma$-globulins under these conditions, the reduction of urea concentration by dilution at pH 7.6, after an alkaline cycle to pH 12.2, results in the recovery of a major portion of the rigidity of the intact molecule (Fig. 8). It is of interest to note that reduction of urea concentration to 2.25 M in alkaline solution (pH 12.3) produces very little recovery of polarization (to 0.102). Evidently the drastic alterations in structure that occur in concentrated urea solution in alkali cannot be reversed unless the stress induced by alkali is relieved. Similar behavior has been noted above with globulin solutions in SDS and TDAC.

The action of 5 M guanidine hydrochloride upon rabbit antibody is quite similar to that of urea except that the steeper decline in polarization occurs above pH 10 (Fig. 10). On back-titratin from 11.25 to 7.0 only minor recovery occurs (Fig. 10). The effect of strong alkali in 5 M guanidine consequently leads to largely irreversible changes in $\gamma$-globulin structure. Similar behavior has been reported in thyroglobulin solutions.

As the level of urea concentration is increased, bovine $\gamma$-globulin rapidly loses its response to...
Fig. 11.—Combined action of urea and detergent upon bovine γ-globulin (BG-V) at 25°. The concentration is 0.05 g./l. O, no SDS; △, 0.004 M SDS. No corrections have been made for solvent viscosity.

SDS (Fig. 11). Indeed, at the highest level of urea (9 M) the addition of SDS appears to increase the polarization slightly. Clearly there is no cumulative effect of SDS and urea.

Qualitatively, the molecular kinetic, rotatory and polarization results are all consistent with a picture of the action of urea and guanidine which has now become widely accepted. Exposure of γ-globulin to high concentrations of urea results in its gradual transition from a compact and rigid particle to a largely unorganized coil. Further discussion of the mechanism of this transition will be postponed until later in the paper.

The Intensity of Fluorescence of DNS Conjugated to γ-Globulins.—The free dye in aqueous media has a pK close to 4.0. When the dye is protonated it loses its fluorescence. It is thereby possible to determine the pK of the dye when it is conjugated to protein. Klotz and Fiess have reported that the pK of DNS (as measured spectrophotometrically) when coupled to bovine serum albumin is depressed by about 2.5 units. In concentrated urea solutions a normal pK was observed. We have observed similar effects with γ-globulins. The pK was found to be about 1.6 and 1.0 for the rabbit antibody and the bovine γ-globulin, respectively (Fig. 12). In 9 M urea the pK of the fluorescent residue was normalized to a value (4.1) close to that for unconjugated dye. However, rather low concentrations (0.012 M) of SDS were equally effective in raising the pK of the residue to its normal value (Fig. 12).

The displacement of the pK of ionization of the dimethylamino group resulting from the presence of the protein appears to be similar in magnitude to that observed by Klotz and Fiess in the case of serum albumin. The return of the pK to a

Discussion

Within the limits imposed by the non-equivalence of the various criteria, the results obtained by the fluorescence polarization technique are basically in harmony with the conclusions attained by other methods. All methods appear to reveal a high degree of lability of the internal structure of both rabbit and bovine γ-globulin with respect to a number of external parameters.

The increase in intrinsic viscosity and frictional ratio occurring at alkaline pH for γ-globulins of either species is accompanied by a definite decrease in β. Almost certainly, all of these reflect the consequences of the partial loss of secondary and tertiary structure occurring under these conditions. This is accompanied by a decline in internal rigidity and by an inflation of the molecular domain. It would be tempting to regard the changes in optical rotation as arising from a loss of α-helical content in addition to the above. However, in view of the considerations developed by Tanford, it is doubtful whether so explicit an interpretation is justified at the present time.

Quantitatively the three molecular criteria are not completely in harmony. Thus the polarization results appear to indicate that a change in molecular structure is occurring at more neutral pH values than those at which the optical rotation begins to change. However, there is nothing in

herently implausible in the idea that the introduction of internal degrees of rotational freedom might precede a loss of structure extensive enough to permit a perceptible change in the configuration of the asymmetric centers. The order of change is: first, a loss of rigidity; next, a molecular inflation detected by viscosity; and finally, a major change in conformation, accompanied by a loss of solubility.

If no artifact is present, the polarization data definitely suggest the occurrence of molecular events too subtle to be reflected by changes in the rotatory or molecular-kinetic properties. These anticipate the grosser changes detected by the latter methods for both the alkaline pH and urea profiles.

In the case of the rabbit antibody virtually all of the initial polarization is recovered even after an hour at pH 11.4. Beginning at about this pH a time-dependent drop in polarization and loss of rapid reversibility become noticeable. These latter phenomena become very important at pH's greater than 12. Indeed the pH dependence of the loss of rapid reversibility appears to parallel the pH profile of solubility to an extent which suggests that the two may be manifestations of the same basic molecular events.

It would be tempting to attribute the combined observations on the alkaline behavior of the rabbit antibody to two distinct processes. The first of these, which begins to be noticeable at pH's above 9, is reflected by a reversible loss of internal rigidity and some molecular inflation but by no major change in the solubility or in whatever molecular features are responsible for the optical rotation. Processes of the second kind, which become important only above pH 11, show a definite time dependence and are not rapidly reversible. They are manifested by a simultaneous change in the fluorescent intensity, optical rotatory, molecular-kinetic and solubility properties of the native molecule.

In the case of bovine γ-globulin the issue of reversibility is confused by the occurrence of very extensive aggregation upon reversal of the alkaline titration, as revealed by the elevated polarizations of fluorescence and by direct ultracentrifugal observation. However, the basic features of the forward process appear to be similar to the rabbit antibody case.

The small drop in relaxation time occurring at acid pH is likewise consistent with the molecular-kinetic changes observed in this region. The gross features of this process appear to be generally similar to those of the alkaline transition, although the change in relaxation time is considerably smaller and complicated by aggregation processes. Moreover, there appears to be considerable dependence upon ionic strength in the acid case possibly brought about by changes in the degree of aggregation. Most of the decrease in $r_0$ is regained upon the return to neutrality in the case of the rabbit antibody. The bovine globulin again shows an important degree of aggregation.

The transitions occurring in the presence of high concentrations of either cationic or anionic detergents are much more drastic than those discussed above. Jirgensons has reported on some interesting observations of the effect of various detergents on the rotatory dispersion constants of serum γ-globulins from various sources. His implication of increase in optical activity in the γ-globulins is not reflected in the polarization data o γ-globulins in TDAC or SDS. The internal structures of both bovine and rabbit γ-globulin appear to be very labile with respect to the action of detergent of either type. Although the limiting relaxation times in excess detergent differ somewhat, the qualitative analogy is complete. In the case of the bovine protein the reversibility of the SDS profile is incomplete, a definite hysteresis appearing between the forward and reverse curves.

The presence of either cationic or anionic detergent, but especially the former, serves in addition to labilize the molecule with respect to alkaline pH. Indeed the relaxation time of rabbit antibody in excess TDAC at pH 12 is so low as to be incompatible with the persistence of any important degree of molecular organization. Among the interesting points which follow from this observation is the conclusion that some fraction of the molecular organization is resistant to TDAC alone and requires the combined action of TDAC and alkali for its elimination.

The structural loss produced by the action of high concentrations of urea likewise appears to be essentially reversible by this criterion. However, in view of the marked hysteresis and incomplete recovery of optical rotation, it is obvious that the recovery of rigidity cannot involve the reestablishment of all features of the initial molecular organization. The parallel between the urea profiles of optical rotation and of polarization is imperfect. While the regions of steepest variation occur at urea concentrations above 4 M in both cases, a definite drop in polarization occurs at levels of urea too low to produce any significant change in rotation. Thus, as in the case of the alkaline profile, an appreciable loss of rigidity appears to precede the extensive disruption of structure which is reflected by a major change in optical rotation and by the inflation of the molecular domain.

The combined action of urea and alkali appears to take γ-globulins of either species almost to the limiting physical state of a random coil. Nevertheless, the ability to regain most of the initial rigidity persists even under these extreme conditions. Thus a return to neutral pH, followed by dilution, results in a progressive approach to a polarization close to the initial.

It is of some interest that, as in the case of thyro-globulin described earlier, there appears to be no cumulative effect of detergent and 8 M urea at neutral pH. Indeed the combined action of 8 M urea and SDS appears to be slightly less effective than urea alone. It is thus unlikely that there exists a class of internal bonds which is resistant to urea but labile to detergent. It is, in fact, rather tempting to speculate that the two agents
may, in actuality, attack similar types of internal linkage.

There appears to be a considerable divergence in action between urea and guanidine hydrochloride, as regards to reversibility of the alkaline pH profile in the presence of high levels of these reagents. The action of guanidine in unfolding proteins is undoubtedly more profound, on a molar basis, than urea. A possible explanation is consequently that S-S bridges may be more exposed to attack by base in guanidine solutions and thereby suffer (irreversible) oxidative cleavage leading to irreversible conformational changes.  