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Structural Transitions in Antibody and Normal γ-Globulins. I. Molecular Properties

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The molecular behavior of bovine and rabbit γ-globulin has been explored by velocity sedimentation, viscosity, optical rotation, solubility, fluorescence and ultraviolet spectrophotometry. All the methods reveal a time-dependent loss in native structure whose rate becomes measurable at pH ~11 and increases rapidly with pH. Only a partial recovery in molecular properties was found on neutralization. 8 *M* urea produced considerable inflation of the molecular domain and an increase in levorotation of the γ-globulins. However, further structural disorganization was produced by alkali (in 8 *M* urea solutions). Where configurational modifications were found by the above methods, changes in fluorescence polarization were always evident. Of interest, however, is that changes in polarization were detectable before any of the more classical methods were able to record any effects. Finally evidence based on alkaline denaturation is presented which would appear to show that a purified rabbit antibody shows the same pattern of heterogeneity as normal rabbit γ-globulin.

At present it cannot be claimed that the γ-globulins are among the proteins which have been well-characterized on the molecular level. The heterogeneous nature of this protein undoubtedly imposes severe obstacles upon studies with the usual techniques.[1-4] Nevertheless the limited data available permit some generalizations as to the behavior of γ-globulins from a variety of mammalian sources.

At low ionic strengths the γ-globulins have limited solubility in the isoelectric region (pH 6.5–7.5). Examination of the material in solution at ionic strengths of 0.01 or less has generally revealed the presence of extensive association.[5,6] This has often been regarded as reflecting electrostatic interactions between oppositely charged components of the γ-globulin solutions. At a higher ionic strength (0.1) the aggregation is largely suppressed.[5,6]

While there is no evidence for any molecular transformation occurring between the pH limits 4 and 9, provided that the concentration of electrolyte is high enough to suppress association, the exposure of γ-globulin to pH's outside these limits appears to result in structural modifications. These are reflected by increases in viscosity,[7-9] frictional ratio[10,11] and levorotation.[7,8]

While the detailed character of the molecular events responsible for these effects is of course unknown, it is possible to make certain rather obvious conclusions. The changes in frictional ratio at extremes of pH are certainly consistent with, and suggest, the occurrence of an inflation of the molecular domain under these conditions. This in turn suggests that the transition to an expandable state may be accompanied or preceded by more subtle changes which result in a partial loss of the rigidity characteristics of the molecule.[12] Moreover, the alteration in molecular-kinetic properties is paralleled by changes in optical rotation which are in the direction expected if a loss in α-helical content occurred.[13,14]

It is the purpose of the present set of papers to examine in detail the molecular state of γ-globulins from two sources under a wide range of conditions. In addition, particular attention has been given to correlating data obtained by fluorescence polarization[12] with other methods as explored in this communication.

Methods and Materials

Physical Measurements.—Ultraviolet spectra were measured with a Beckman DU spectrophotometer, equipped with a photomultiplier attachment. Ultracentrifuge measurements were made with a Spinco Model E ultracentrifuge.

Viscosity determinations were made with a low shear viscometer of long solvent time (300 seconds for water). A Radiometer Model TTTl pH meter, equipped with Miniature Leeds and Northrup electrodes, was used to measure pH. The instrument was standardized with Beckman buffers of pH 4.00, 7.00 and 10.00. In the spectrophotometric titration experiments γ-globulin (~0.5%) in 0.20 *M* KCl was diluted about 5-fold into a 0.20 *M* KCl–0.015 *M* lysine solution. Approximately 2.5 ml. samples were titrated in 1 cm.^{2} quartz cuvettes with very small amounts of either 2 *M* HCl or KOH which were delivered through a polyethylene catheter from a Agla precision syringe. Solutions were mixed by magnetic stirring. In this way a pH curve could be obtained on a single sample. Measurements of fluorescence intensity were made with an Aminco–Bowman spectrofluorometer, modified to permit temperature control.

Solubility Measurements.—Considerable caution must be exercised in the choice of buffer that is used to quench the denaturation reaction. It was initially found that partial precipitation of denatured rabbit or bovine γ-globulin could be brought about by standing for several hours in 0.4 *M* phosphate, pH 6.8. Much more rapid and quantitative precipitation occurred if more concentrated phosphate and a protein level not lower than 0.25% were used. The usual quenching procedure was to mix equal volumes of globulin solution and phosphate buffer. A molarity of 4.5 was found to be optimal for the latter. Lower molarities (4.25 or less)

(1) R. A. Alberty, *J. Am. Chem. Soc.*, **70**, 1675 (1948); *J. Phys. Colloid Chem.*, **52**, 217, 1345 (1948); **53**, 114 (1949).

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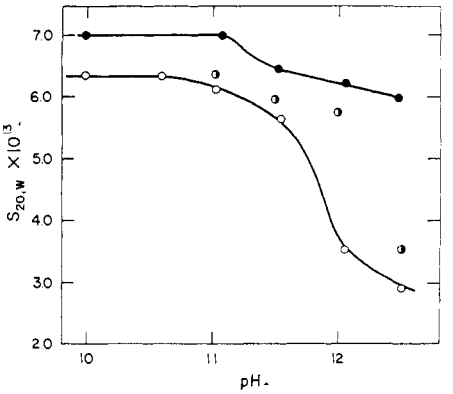

Fig. 1.—Influence of alkaline pH on the sedimentation coefficient of γ-globulins. ⊙, bovine γ-globulin in 0.2 *M* KCl, 0.025 *M* lysine, conc. = 0.50%; ●, rabbit antibody in 0.1 *M* NaCl, 0.035 *M* lysine, conc. = 0.20%; ◑, rabbit γ-globulin in 0.1 *M* NaCl, 0.035 *M* lysine, conc. = 0.50%.

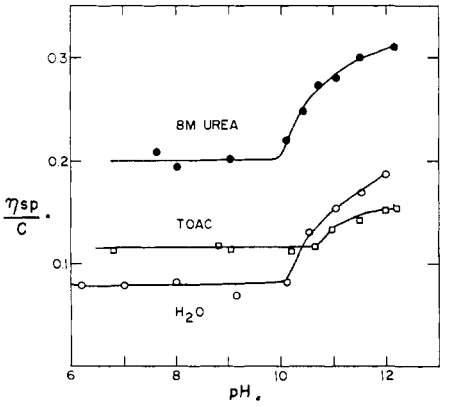

Fig. 2.—Influence of alkaline pH on the reduced specific viscosity of bovine γ-globulin in various solvents. Protein concn. = 0.18%; *T* = 25.0°: ⊙, 0.02 *M* KCl, 0.02 *M* lysine; □, 0.04 *M* TDAC; ●, 8 *M* urea, 0.02 *M* KCl, 0.02 *M* lysine.

failed to precipitate the denatured protein maximally; higher molarities (5.0 or more) precipitated some native globulin.

The precipitate was centrifuged down by 10 minutes spinning in a Sorvall centrifuge at 2000 g. An aliquot of the supernatant was diluted with 8–10 volumes of 2 *M* KOH and its absorbancy measured at 287 mμ. An extinction coefficient of 13.8 was used for a 1% solution of bovine γ-globulin in the latter solvent at 287 mμ.

Materials.—Rabbit and bovine γ-globulins were obtained from Pentex Incorporated and from Armour, respectively. Apart from a small (<5%) fraction of more rapidly sedimenting material, all of both preparations sedimented as a single homogeneous peak with $S_{20} \cong 7$ svedbergs. Rabbit anti-thyroglobulin antibody was prepared from antisera by the method of Metzger and Edelhoch.[15] Sodium dodecyl sulfate (SDS) was a purified preparation donated by E. I. du Pont de Nemours and Company. Trimethyldodecyl ammonium chloride (TDAC) was obtained from Armour and Company. Urea was recrystallized from ethanol. Salts were reagent grade. Glass-redistilled water was used in preparing all solutions. The preparation of fluorescent conjugates of γ-globulin with 1-dimethylamino-naphthalene-5-sulfonyl chloride (DNS) is described in the companion paper.[12]

Experimental Results

I. Influence of pH in Aqueous Media A. Molecular-kinetic Properties.—As seen in Fig. 1 the sedimentation coefficients of γ-globulins of either species and rabbit anti-

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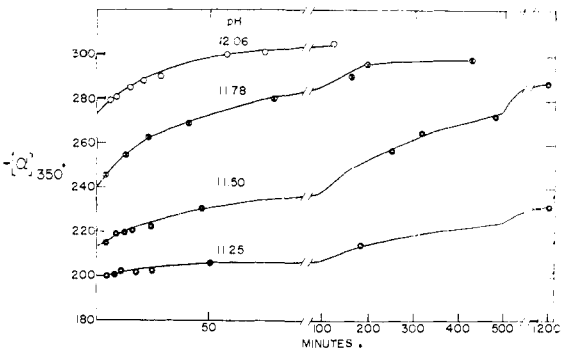

Fig. 3.—Influence of alkaline pH on the rate of increase in levorotation at 350 mμ of bovine γ-globulin (0.5%). Solutions containing 0.10 *M* KCl, 0.10 *M* K_2HPO_4 were adjusted with KOH to indicated pH; *T*, 25°.

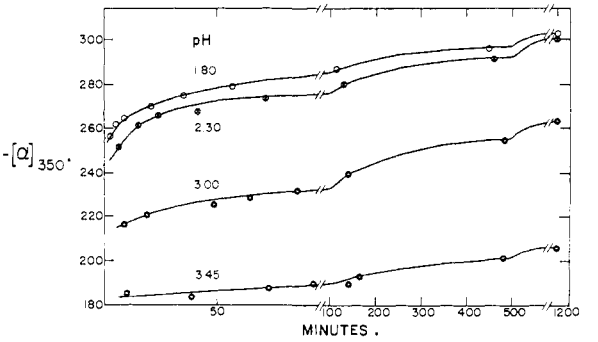

Fig. 4.—Influence of acid pH on rate of increase in levorotation at 350 mμ of bovine γ-globulin (0.50%). Solutions containing 0.2 *M* KCl, 0.015 *M* lysine were adjusted with HCl to the indicated pH; *T*, 25°.

body in dilute KCl showed no important change until about pH 11. At higher pH's a decrease in S_{20} occurred. The change was somewhat more drastic in the case of the bovine protein. There was no resolution into discrete components in either case, although boundary spreading was enhanced.

A definite species difference appeared to exist with regard to reversibility. A return to pH 9.5 after a five minute exposure at pH 12.1 in the case of the rabbit antibody resulted in the reconversion of almost half of the material into a component of sedimentation rate close to that of the original. The balance appeared as poorly resolved, rapidly sedimenting aggregates. In the case of the bovine protein not more than about 10% regained the sedimentation coefficient of native γ-globulin. At the intermediate pH of 10.6 aggregation was somewhat less pronounced and about 80% of the rabbit and 50% of the bovine γ-globulin had sedimentation properties characteristic of their native forms. Allowing the reversed solution to stand for 20 hr. had little effect on the degree of reversal. Similarly, variation of ionic strength between 0.02 and 0.20 appeared to exert no significant influence.

The effect of pH on the reduced specific viscosity of bovine γ-globulin in 0.02 *M* KCl is reported in Fig. 2. The viscosity did not vary between pH 6 and 10. Above pH 10 a pronounced increase was observed. The change in viscosity precedes that of sedimentation but lags behind that of the pH-profile of fluorescence polarization reported in the accompanying paper.[12] The difference between the pH profiles of viscosity and sedimentation probably arises from the greater sensitivity of the former.

B. **Optical Rotation.**—The effect of alkaline pH on the rate of increase in levorotation of bovine γ-globulin in 0.1 *M* KCl–0.1 *M* KH_2PO_4 is illustrated in Fig. 3. The data reveal a number of noteworthy features. It is evident that rotatory changes commence only above pH ~11 and therefore, in this instance, are less sensitive as an indicator of molecular rearrangement than either viscosity or polarization of fluores-

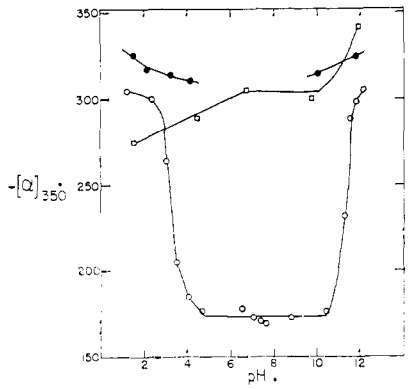

Fig. 5.—Influence of pH on the specific rotation of bovine γ-globulin solutions (0.50%). ⊙, 0.10 *M* NaCl for pH 4.0–10.4; 0.2 *M* KCl, 0.015 *M* lysine at other pH's (taken from Figs. 3 and 4 at 1200 minutes); ●, heated to −70° for four minutes in 0.10 NaCl (measured at 25°); ⊡, 0.03 *M* SDS.

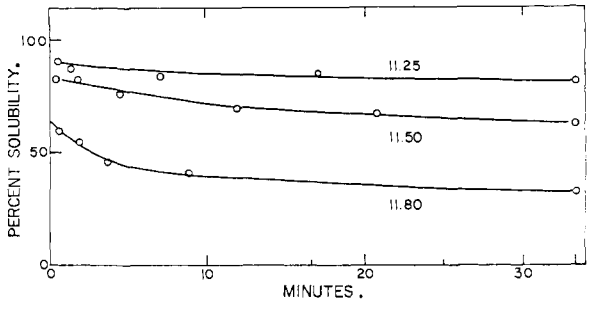

Fig. 6.—Influence of alkaline pH on the rate of denaturation of bovine γ-globulin in 0.20 *M* KCl, 0.025 *M* lysine. Protein concn. = 0.50%; *T*, 25.0°C.

cence data. At higher pH's an initial rapid increase in specific levorotation occurs, whose magnitude is strongly dependent on pH. Subsequently, a further time-dependent increase takes place which appears to approach a limiting value which increases with increasing pH.

Figure 4 reports the kinetics of the levorotatory change of bovine γ-globulin in 0.2 *M* KCl at pH 1.80 and 2.30. The limiting values of specific rotation at these pH's are in close agreement with those found at 11.78 and 12.06. At pH 3.00 a rapid initial increase occurred, followed by a slow change which did not reach the value observed at pH 1.80 even after 20 hr. At pH 3.45 only a very slow rate was recorded. As with the alkaline data the earliest perceptible changes in polarization of fluorescence occur at somewhat more neutral pH values than in the case of specific rotation.

Consistent with the only partial recovery of the initial sedimentation pattern of γ-globulin, after exposure to pH 12.1 for five minutes, is the incomplete reversibility, as determined by optical rotation. Values close to −255° were observed for $[\alpha]_{350}$ after several minutes, when the pH was decreased from 12.1 to 11.0. At pH 10.1, $[\alpha]_{350}$ returned to −235° in ten minutes and showed no subsequent change. When the pH was taken directly to 9.2, a smaller recovery was observed, in that the rotation decreased only to −250°. Reversal of pH to 3.7 after 5 minutes at 1.9 resulted in only a very small recovery in optical rotation.

This lack of major reversibility observed after exposure to extremes of pH is also seen after heating solutions of bovine γ-globulin to ~70° for about 5 minutes. Between pH 4 and 10 the protein precipitated from solution, precluding any optical rotatory observations. Outside this range the observed values were slightly more levorotatory than those found at 25°. The levorotation increased slightly with increasing acidity or basicity. These data are reproduced in Fig. 5 along with the rotation data observed at 25° after 20 hr.

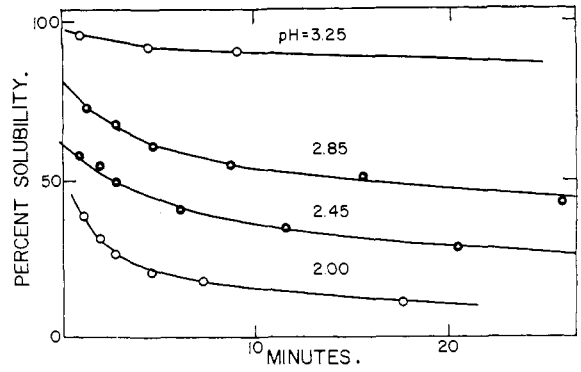

Fig. 7.—Influence of acid pH on the rate of denaturation of bovine γ-globulin in 0.20 *M* KCl, 0.025 *M* lysine. Protein conc. = 0.50%; *T*, 25.0°.

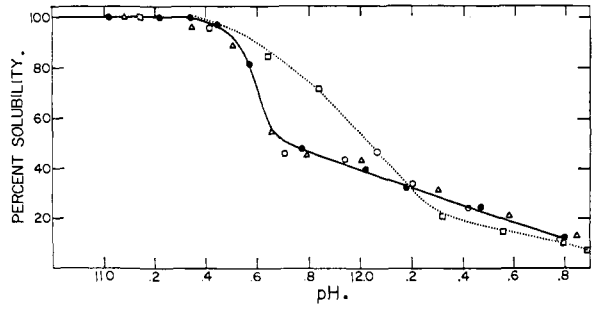

Fig. 8.—Influence of alkaline pH on the denaturation of γ-globulin. All solutions contain 0.10 *M* KCl, 0.35% protein. The procedure was identical for each experiment (see text for details): ⊙ and ●, rabbit antibody, obtained from single animal (AQ-25, see ref. 15). Open and filled circles were two independent experiments; △, rabbit γ-globulin; □, bovine γ-globulin; *T*, 25°.

C. Denaturation.—Denaturation will be interpreted in this communication exclusively in its classical sense: as the loss in solubility that occurs in γ-globulin at or near its isoelectric point. The rate of denaturation in alkaline solution in 0.2 *M* KCl is shown in Fig. 6. It is abundantly clear that the data at the various pH values cannot represent a simple process such as the single step denaturation of a homogeneous substance. All the kinetic curves tend to plateau at different degrees of denaturation depending critically on the pH. In addition, at pH values above 11.25, the curves do not extrapolate to 100% undenatured protein at zero time.

It is of interest to note that the over-all character of the solubility curve, with respect to the pH-dependence of the relative rate, is in rather close accord with the rotatory data. The changes that are evident from polarization and viscosity at lower pH's presumably reflect minor and reversible changes in protein conformation.

The reversal of pH to 10.2 after exposure of bovine γ-globulin to pH 12.1 for 5 minutes produced about a 20% increase in solubility which occurred within 20 minutes.

The denaturation of bovine γ-globulin in 0.2 *M* KCl by acid is reported in Fig. 7. Again the close similarity of the solubility to the optical rotatory data is immediately apparent.

Since rate curves which covered the complete range of loss in solubility could not be obtained at a single pH a different procedure was used to obtain the pH profile of the loss in solubility. In this method the pH was rapidly adjusted to a given value and an aliquot of solution was removed and quenched *immediately* (<10 seconds) with standard precipitating buffer (see "Methods"). This process was repeated at a series of increasingly alkaline pH's. Since the rate of loss in solubility at a single pH is rather low after the initial "instantaneous" loss, and the process is very incomplete (see Fig. 6) a "pH-solubility curve" was obtained by

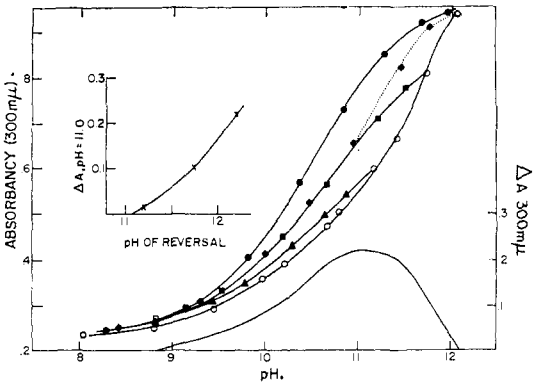

Fig. 9.—The spectral titration at 300 mμ of bovine γ-globulin in 0.2 *M* KCl, 0.015 *M* lysine; O, forward; ●, reverse, from pH 12.25; ■, reverse, from pH 11.75; ▲, reverse, from pH 11.20; ◆, second forward curve after an alkaline cycle to pH 12. Lower inset, difference curve between forward and reverse curve to pH 12.25; Upper inset (X), difference in absorbancy between forward and reverse curves at pH 11.0 as a function of the pH at which reverse curves were initiated. Protein conc. = 0.11%; T = 25.0°.

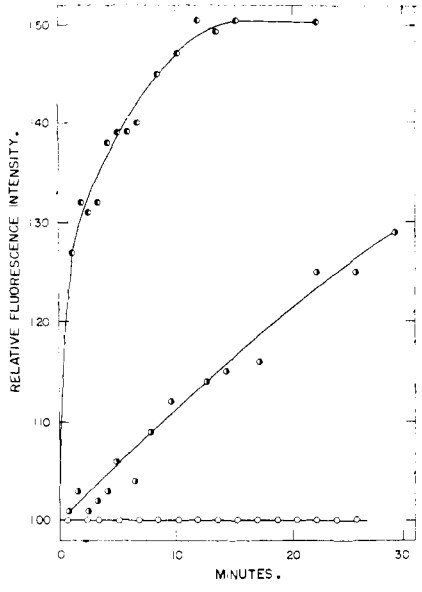

Fig. 10.—Increase in relative fluorescence intensity with time for a DNS conjugate of bovine γ-globulin (BG-VI, see ref. 12) at several alkaline pH's in 0.02 *M* NaCl, 25°. The concentration is 0.05%. ⊙, pH 11.3; ◑, pH 11.8; ◐, pH 12.4.

this somewhat arbitrary procedure. However, when curves were obtained for rabbit antibody, rabbit γ-globulin and bovine γ-globulin under identical conditions some interesting comparisons emerge. As seen in Fig. 8 the data for rabbit antibody and rabbit γ-globulin fall on a single curve which is significantly different from that of bovine γ-globulin.

D. Spectrophotometric Titration of Tyrosyl Residues.—In view of the preceding information, as well as results upon other proteins, it comes as no surprise to find a markedly abnormal character for the ionization of the tyrosines. The titration data for the forward (adding KOH) and reverse (adding HCl) curves, *i.e.*, from 11.18, 11.75 and 12.2, are shown in Fig. 9. Thus the apparent pK of tyrosine, as determined from the spectral titration, is displaced toward lower values by exposure to a pH above 11. Moreover, the divergence of the forward and reverse branches of an alkaline cycle to pH 12.2 is such as to indicate that an important

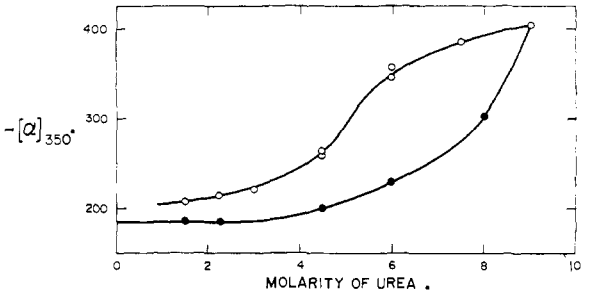

Fig. 11.—Effect of urea concentration on the specific levorotation of bovine γ-globulin at pH 7.0 in 0.05 *M* NaCl and 0.05 ionic strength phosphate buffer: ●, forward curve; O, reverse, from 9 *M* urea.

change in the state of the tyrosines accompanies the exposure of the protein to alkaline pH values. A difference plot (see bottom curve, Fig. 9) of the forward and reverse curves shows a maximum at about pH 11.

If the reverse half-cycle is begun at a series of pH's of increasing alkalinity, and the vertical displacement plotted against the pH at which reversal was initiated, it is seen that the onset of irreversibility also occurs at about pH 11 (see upper inset, Fig. 9). The correspondence of this with the pH region at which the change in optical rotation and loss in solubility begins is striking. It is also evident that no break or inflection occurs in the titration curve to mark the onset of irreversibility. About 40% of the total change in absorption at 300 mμ is complete at pH 11.0. It is not possible with the γ-globulins to distinguish tyrosyl residues of different stability by their titration characteristics since irreversible structural modifications occur in the pH region of their titration.

A *second* forward titration was performed on a solution immediately after it had been titrated to 12.1 and then back-titrated to 8.4. This data is recorded also in Fig. 9 and shows a partial recovery towards the first forward curve. This result is in qualitative accord with the partial reversal observed by the methods already discussed.

E. Intensity of Fluorescence of Conjugates.—The polarization of fluorescence of DNS conjugates of rabbit and bovine γ-globulin is discussed in detail in the companion paper.[12] In addition to the effects considered elsewhere, there occurs a definite time-dependent exaltation of fluorescence intensity at alkaline pH (Fig. 10). This process begins to be apparent at a pH alkaline to 11.3. Its rate increases very rapidly with pH so that the change is almost instantaneous at pH 12.4. The enhancement of intensity is not reversed upon back-titration to neutral pH. This effect appears to parallel, at least roughly, the other time-dependent phenomena which begin at a pH close to 11. There can be little doubt that they all represent different facets of the drastic molecular changes occurring under these conditions. Thus the intensity of fluorescence of the DNS label is by no means entirely insensitive to its environment. The detailed mechanism of the interdependence must remain open for the present.

II. Influence of Urea.—The exposure of bovine γ-globulin to increasing molarities of urea at pH 7.0 has only a minor effect on its optical rotation up to molarities of about 6. Beginning at this level there occurs a rapid elevation in $-[\alpha]_{350}$ as is seen in Fig. 11. The values shown in Fig. 11 were obtained immediately after addition of urea.

When the urea concentrations of the above solutions were reduced from 9 *M* by dilution with solvent, most of the change in specific rotation was ultimately recovered. However, a major degree of hysteresis appeared between the forward and reverse branches (Fig. 11). Examination of bovine γ-globulin in the ultracentrifuge after dilution from 9 *M* to 4.5 *M* urea at pH 7.0 showed extensive aggregation. On raising the pH from neutrality to 12 in 8 *M* urea a dramatic increase in levorotation was found, as is reported in Table I. Rabbit γ-globulin showed the same change in rotation when the pH was raised in 8 *M* urea.

Bovine γ-globulin at neutral pH shows a pronounced rise in viscosity when the urea concentration is increased to 8 *M* (Fig. 2). That a considerable fraction of the internal organization of the molecule must persist in 8 *M* urea at

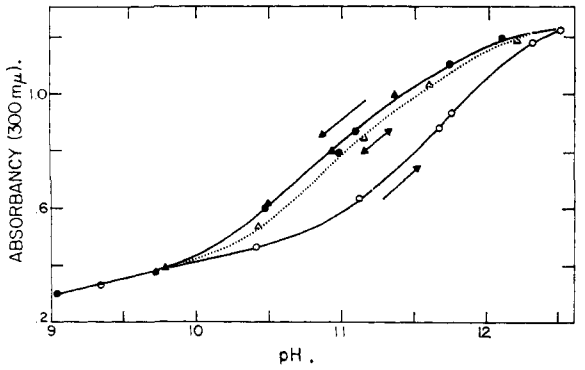

Fig. 12.—The spectral titration at 300 mμ of bovine γ-globulin (0.13%) in 8.7 *M* urea, 0.013 *M* NaCl, 25°: ⊙, forward; ●, reverse, from pH 12.5; - - - and Δ, second forward, from pH 8.6 after alkaline cycle to pH 12.5; ▲, second reverse, from pH 12.1.

neutrality is apparent from the pH dependence of optical rotation and viscosity in this medium. The influence of alkaline pH on the reduced specific viscosity is shown in Fig. 2. A profound increase is seen to occur above pH 10 and appears to attain completion at pH ~12. The total change is sufficiently great to reveal a major inflation of the molecular domain. Thus, most or all, of the alkaline conformational change does not appear to have been anticipated by this level of urea.

TABLE I

SPECIFIC ROTATION OF BOVINE γ-GLOBULIN[a]

pH	Salts	Reagent	$-[\alpha]_{550}$	$-[\alpha]_{350}$
7.0	0.02 *M* KCl	8 *M* Urea	95	305
12.0	.02 *M* KCl	8 *M* Urea	130	430
7.0	.02 *M* KCl	0.05 *M* TDAC	57	190
12.0	.02 *M* KCl	.05 *M* TDAC	66	235
7.0	.....	.03 *M* SDS	82	305
12.0	.....	.03 *M* SDS	95	335
1.5	.....	.03 *M* SDS	72	275
7.0	.10 *M* NaCl	.....	54	170

[a] Values uncorrected for solvent refractions. T = 25°. Protein concentration = 0.35%. Very similar rotatory changes were observed for rabbit antibody in 8 *M* urea and 0.05 *M* TDAC.

Additional evidence for a further structural change at alkaline pH values in concentrated urea solutions came from the nature of the spectral titration curve of the tyrosyl residues as reported in Fig. 12. Not only does a definite abnormality persist in 8.7 *M* urea but there is also an important divergence between the forward and reverse curves. The two curves are mutually displaced by 0.6 pH units at their midpoints. A second forward curve begun from pH 8.6, after an alkaline cycle to pH 12.5, is only slightly displaced from the initial *reverse* curve. A second reverse curve from pH 12.2 falls on the first *reverse* curve. The transition that occurs in alkali is therefore either irreversible or encounters a strong kinetic barrier to reversal at pH 8.6 or above.

There is no appreciable difference in titration behavior between bovine and rabbit γ-globulin under the conditions stated in Fig. 12.

III. Influence of Detergents.—As in the case of high levels of urea a considerable fraction of the internal structure appears to persist in the presence of excess detergent at neutral pH. A small increase in reduced specific viscosity occurs in excess TDAC at neutral pH values as shown in Fig. 2. At about pH 10.7 the viscosity begins to increase and appears to level off near pH ~12. At the latter pH the viscosity in TDAC is less than that in water. Consequently less inflation appears to take place at alkaline pH values in the presence of TDAC. Optical rotatory data are completely consistent with those from viscosity. As recorded in Table I, only a quite small increase in $-[\alpha]_{350}$ occurs in 0.05 *M* TDAC at neutral pH. On raising the pH to 12 the levorotation increases considerably but falls short of the value found in water.

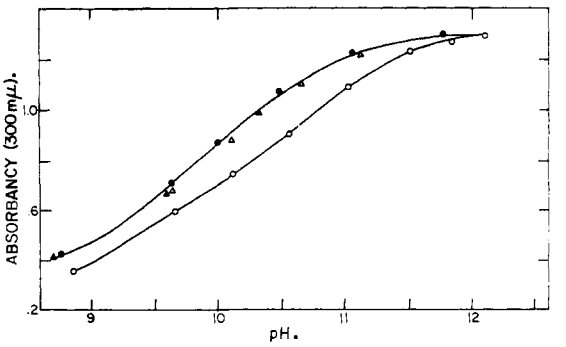

Fig. 13.—The spectral titration at 300 mμ of bovine γ-globulin in 0.022 *M* TDAC, 0.013 *M* NaCl. Protein conc. = 0.13%. T = 25°. ⊙, forward; ●, reverse, from 2.1; Δ, second forward from pH 8.7 after alkaline cycle to pH 12.1; ▲, reverse curve from pH 11.1.

The spectral titration curve in 0.02 *M* TDAC (0.013 *M* NaCl) also shows a major hysteresis, of about 0.5 pH units at the midpoints, between the two halves of an alkaline cycle to pH 12, as is seen in Fig. 13. The midpoint of the forward curve occurs at pH ~10.5, revealing a marked acid shift from that observed in water. This increase in acid strength of the phenolic residues presumably stems, at least in part, from a reduction in the electrostatic component of the free energy change accompanying ionization, as a result of the combined effects of over-all charge neutralization and protein unfolding accompanying the binding of detergent. A second forward curve showed a slight displacement from the first *reverse* curve.

Somewhat similar behavior is encountered with the anionic detergent SDS; however, in this case the structure of bovine γ-globulin is less disorganized at low pH than at neutrality (Table I). As appears in Fig. 5 the optical levorotation of bovine γ-globulin in 0.03 *M* SDS is somewhat less at pH 1.5 than at neutrality. These effects are explicable in terms of a reduction in net charge of the protein due to detergent binding. Cross-linking by bound detergent may also play a role in stabilizing the unfolded molecule. When the protein and SDS are similarly charged as at alkaline pH, maximum disruption of internal organization may occur as is seen by the further increase in $-[\alpha]_{350}$ at pH 12 (see Fig. 5). Further evidence for the SDS-induced unfolding of γ-globulin at neutral pH is the reduction of sedimentation coefficient under these conditions (Table II).

TABLE II

SEDIMENTATION COEFFICIENTS OF BOVINE γ-GLOBULIN IN SDS[a]

Molarity SDS	$S_{20,w}$
0.01	5.46
.02	4.49
.03	4.40

[a] Protein concentration = 0.35%; NaCl = 0.02 *M*. Above 0.01 *M* SDS the detergent boundary was evident in the schlieren pattern and the boundaries showed artificial sharpening.

Discussion

The investigation reported herein of certain properties of the γ-globulins was deemed necessary to confirm and extend the results obtained by the polarization of fluorescence study described in the accompanying paper.[12] In all instances where molecular alterations are found by hydrodynamic, spectrometric or polarimetric measurements, concomitant changes are observed by polarization. In fact the latter technique can often detect incipient changes in structure where the hydrody-

namic procedures show little or no modification. Since polarization measurements are essentially instantaneous and require relatively small amounts of labelled protein, it may well be the method of choice in detecting structural modifications in proteins.

It is clear from the above data that there is a virtually continuous transition from those properties characteristic of the native molecules to those of the almost totally disorganized conformation prevailing under the most drastic conditions examined. Nevertheless, in the case of the molecular changes induced by alkaline pH, at least four quite different criteria are in harmony in indicating that a critical extent of ionization occurs at about pH 11. Below this critical pH no *irreversible* changes have occurred, as is indicated by the absence of any loss in solubility. Moreover, the optical rotation is unchanged as is also the intensity of fluorescence of a DNS conjugate. Beyond this limit there occurs a time-dependent and incompletely reversible change in *all* of the above. Kleinschmidt and Boyer reported a decrease in specific precipitate formation in an immune rabbit γ-globulin after exposure to pH 12.3, whereas pH 11.7 had almost no effect.[16]

The viscosity results indicate that the major loss in molecular organization occurring at pH $\sim$11 is preceded, at least at low ionic strength (0.01), by some degree of deformation of the molecular domain. Apparently some distortion is allowed by the intact molecule without resulting in any irreversible loss in structure. The decrease in polarization of fluorescence begins at somewhat lower pH values than does the increase in viscosity, indicating that a reduction in relaxation time precedes a change in hydrodynamic volume.[12] It would thus appear that an increase in internal rotation due to bond rupture can be detected by the polarization technique prior to the point where the disorganization is reflected by hydrodynamically detectable shape changes.

Most of the change in optical rotation at neutral pH occurs at levels of urea greater than $\sim$5 M. That some structure persists even in 8 M urea at neutral pH is indicated by the major degree of pH dependence shown by optical rotation, molecular-kinetic properties and by hysteresis of the spectral titration curve. In contrast to the fluorescence polarization results of the companion paper,[12] an important degree of hysteresis exists between the forward and reverse branches of the urea profile of rotation at neutral pH.

The molecular disorganization induced by detergent, either cationic or anionic, likewise fails to approach completion at neutral pH. That a further loss in structure occurs at more alkaline pH is shown by the elevation in rotation and viscosity under these conditions.

The difference between the actions of SDS and TDAC in neutral and alkaline solution is noteworthy. The average isoelectric point of the γ-globulins is not far from neutrality.[1] In consequence, the constellations of positive and negative charges should be similar at neutral pH. As a result, the purely electrostatic factors involved in the binding of anionic SDS or cationic TDAC would be expected to be likewise similar. However, increasing degrees of molecular disorganization, without precipitation, are observed for all SDS: protein ratios at neutral pH, in contrast to the broad precipitation zone observed for a similar range of TDAC levels. At sufficiently high TDAC levels redissolution occurs, yielding a solution of modified globulin, whose molecular state is comparable to that found for high SDS levels. This behavior is rather reminiscent of that observed with thyroglobulin.[17]

It is thus quite clear that those molecular conformational changes produced by alkaline pH in aqueous media persist at least in part, or in their entirety, in concentrated urea or detergent solutions in spite of the marked effects on structure produced by these latter reagents. The alkaline pH-labile sector of the molecular organization of the γ-globulins appears therefore to be refractory to both urea and detergents. Consequently it cannot be presumed that all the functional groups in a protein will show their normal reactivity in these solvent systems. The tyrosyl groups of lysozyme have recently been shown to titrate irreversibly in 9 M urea.[18] Judging by the relatively high values of polarization (and rotational diffusion constant) of thyroglobulin in 9 M urea or 5 M guanidine, at neutral and even alkaline pH values, it seems likely that considerable internal structure persists in this protein under these conditions also.[17]

The evidence for the molecular heterogeneity of the γ-globulins is overwhelming. This heterogeneity in electrophoretic[1] and chromatographic[19,20] properties is well known to extend to antibodies directed against a single protein antigen and even to antibodies against a single hapten group.[21]

The kind of kinetics obeyed by the incompletely reversible processes occurring at pH's alkaline to 11 is consistent with the view that these systems are heterogeneous with respect to susceptibility to structural change as well. If the pH profile of alkaline denaturation is a valid criterion of this latter heterogeneity, then the results of this study serve to reinforce the conclusion that purified antibodies are indistinguishable in this respect from normal γ-glogulin. If this is the case, the task of reconciling this absence of discrimination in the formation of immune globulins with the extreme selectivity conferred upon the active sites is a challenging one.

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