Alkali Degradation of Rabbit γG-Immunoglobulin*

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ABSTRACT: The degradation of rabbit γG-immunoglobulin by alkali at pH values between 12.0 and 12.4 has been followed by ultracentrifugation, viscometry, light scattering, spectrophotometric titration, and sulfhydryl determinations. It was found that the molecule first swells and then dissociates into half-molecules at a rate dependent upon the temperature. The spectral curve of titratable tyrosines showed a pronounced shift during this degradation, and this shift was entirely accounted for by the change in the electrostatic interaction parameter, w, occasioned by the swelling and subsequent dissociation. Antibody activity was apparently lost when the molecule dissociated into half-molecules above pH 12, and attempted recombination experiments were unsuccessful. An unexpected result was the finding that no new sulfhydryl groups appeared during the dissociation reaction until approximately one-half of the molecules had been split in two. Dissociation of γ-globulin into half-molecules by alkali was markedly inhibited by preincubation with iodoacetamide in the presence of detergent. To account for this we suggest that an intramolecular sulfhydryl–disulfide-interchange reaction is responsible for the cleavage of the disulfide bridge linking the two half-molecules.

γG-Immunoglobulin is split into subunits by both acid and also alkali treatment. The protein may be split into half-molecules by reduction of the disulfide bonds, followed by dialysis against low ionic strength buffers of pH less than 2.5 (Palmer et al., 1963). Reduction of only one disulfide bond per mole of intact globulin appears to be sufficient for dissociation of one-half to two-thirds of the molecules. Later these authors showed (Nisonoff and Palmer, 1964) that the halves would combine when the pH was returned to neutrality, and that hybrids could be formed from halves of distinct antibodies. This recombination occurs even though the fragments have been alkylated, indicating that the linkage in the recombined molecule is by noncovalent interaction only. On the other hand, if the alkylation step were omitted, a disulfide linkage was re-formed between the recombined halves (Stein et al., 1964).

Jirgensons et al. (1963) studied the denaturation and subsequent fragmentation of γG-immunoglobulin by alkali in the presence and in the absence of detergent. In the presence of detergent, they reported that the molecule was unfolded in alkaline solutions of pH 10.2–11.0, and then split into fragments having molecular weights of approximately 60,000. Edelhoch et al. (1962) also showed that alkali causes a decrease in the sedimentation coefficient and an increase in viscosity. They noted a difference in the forward and reverse spectral titration of phenolic groups.

In this paper are described the results of studies which we have made upon the degradation of rabbit γG-immunoglobulin by alkali. The changes induced by alkali are followed by sedimentation, viscosity, and light-scattering measurements, spectrophotometric and amperometric titrations, and antigenic and antibody analysis. We find that the molecules in alkali first swell, and then dissociate into half-molecules without the appearance of new sulfhydryl groups. This dissociation can be inhibited by pretreatment with detergent and iodoacetamide. Therefore, we believe that a sulfhydryl–disulfide exchange is involved in the splitting reaction. In addition, we present evidence that the changes in the spectrophotometric titration up to pH 12.4 can be accounted for by the swelling of the molecule and subsequent dissociation into half-molecules, without postulating an exposure of buried tyrosyl residues.

Materials and Methods

Preparation of γ-Globulin. The γ-2-globulin fraction of serum proteins were used with all experiments described in this paper. The globulin was isolated from rabbit serum by ammonium sulfate fractionation followed by chromatography on DEAE-cellulose in a manner similar to that described by Utsumi and Karush (1964).

The final preparation shows one component by electrophoresis at pH 8.5 in 0.1 M Veronal buffer, and by ultracentrifugation, except for a small contamination, less than 5% at the leading edge. Most of the
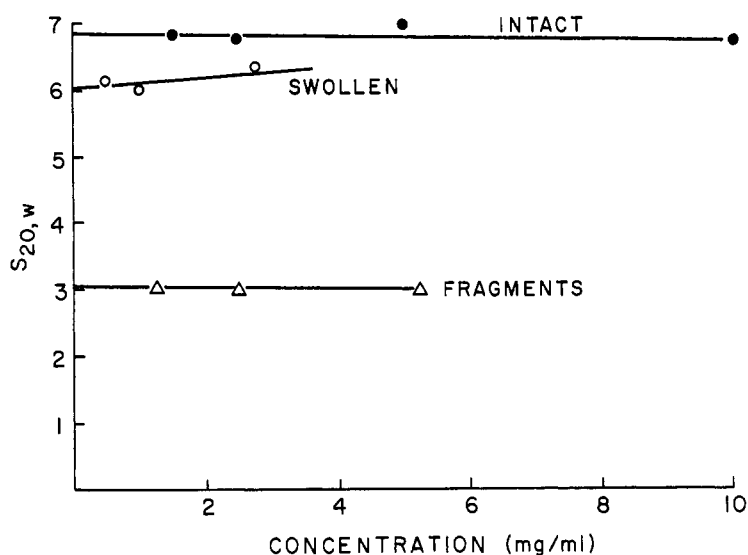
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FIGURE 1: Sedimentation coefficients of γ-globulin at pH 6.5 (intact), 12.0–12.4 (swollen), and 12.4 (fragments). See Table I for buffer composition. The "swollen" molecules were studied immediately after raising the pH, while the fragments were studied 30–60 hr later. All runs were made at 4° at 59,780 rpm.

experiments described have been repeated using a second preparation of γ-globulin.

Viscometry. Two types of capillary viscometers were used. One was a single-bulb Ostwald viscometer and the other an Ubbelohde dilution viscometer. The low-temperature studies were performed in a water bath containing an ice compartment and a circulating pump. For concentration-dependent studies, dilutions were made in the viscometer itself.

Solutions were taken directly from the viscometer and placed in the centrifuge cell, following the viscosity readings. Alkaline solutions were kept at 0° at all times. The pH was raised by the addition of 0.5 N KOH–0.5 M KCl–0.015 M lysine, with constant stirring.

Analytical Ultracentrifugation. All runs on solutions of a pH higher than 9 were performed in filled-Epon cells to avoid reaction of alkali with duralumin. Most of the velocity runs were made at 59,780 rpm. A few were performed at 56,100. All sedimentation coefficients are corrected to 20°. Equilibrium sedimentation measurements were made using a six-channel "Yphantis" cell, purchased from Spinco Division of Beckman Instrument Co. Fluorocarbon was layered at the bottom of each fluid column. The reference solvent was always the diffusate, after dialysis of the solution. Runs were made at 12,590 rpm.

Light Scattering. Light-scattering measurements were made with a Phoenix Precision Instrument Co. light-scattering photometer, linked to a Sargent automatic recorder. An alkali-resistant Pyrex cell was used for all studies at high pH. All measurements were made at 90° angle with rectangular cells of 3–4-ml capacity.

Sulfhydryl Determinations. Sulfhydryl assays were done by amperometric titrations with $AgNO_3$, according to the method of Benesch *et al.* (1955). The

titrations were carried out on a Sargent manual polarograph Model III attached to a rotating platinum electrode and a mercuric oxide electrode, as described in the above reference. Equal increments of 4×10^{-3} M $AgNO_3$ were added at equal time intervals of 1 or 2 min, by means of a syringe microburet (Micro-metric Instrument Co.). In the majority of experiments, a potential of 0.24 v was applied across the electrode.

TABLE I: Sedimentation, Viscosity, and Sedimentation Equilibrium Measurements for Rabbit γ-Globulin as a Function of pH.

Treatment	$s_{20,w}^0$	$[\eta]$	$M_{\eta,s}$	$M_{se}{}^a$
0.15 M KCl–0.015 M lysine (pH 6.5)	6.79	0.070	158,000	147,000
0.15 M KCl–0.015 M lysine + KOH (pH 12.0–12.4) (fast component)	6.05	0.097	156,000	—
0.05 M Tris (pH 11.0)	—	—	—	147,000
0.15 M KCl–0.015 M lysine + KOH (pH 12.4) (slow component)	3.45	0.097	66,900	—
0.05 M K_2PO_4 (pH 12.4)	3.05	0.141	68,400	—

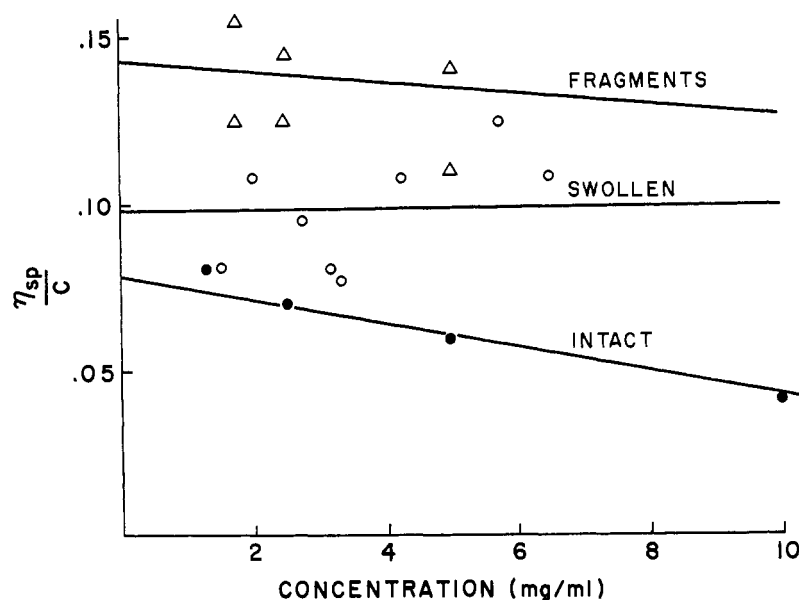a se = sedimentation equilibrium.

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FIGURE 2: Viscosities of γ-globulin. See legend of Figure 1 for details.

Between experiments a small amount of the KNO_3 from the salt bridge was allowed to flow through the porous disk of the electrode, and both electrodes were rinsed with glass-distilled water. A time lapse of at least 5 min was allowed after the flow had been stopped for pressure equilibration in the tubing. The electrodes were again rinsed, dried, and the next titration was begun. The solvent for the titrations had the following composition: 40 ml of 1 M Tris, 34 ml of 1 M HNO_3, 3 ml of 1 M KCL, 30 ml of 10% sodium lauryl sulfate, and 183 ml of deionized glass-distilled water. The total volume of a given titration solution was 30 ml, of which 0.5–2.0 ml was sample, and the remainder solvent. Assays of globulin employed 0.1–0.5 μmole of globulin for their titration. Solvent blanks were always run and always gave values of less than 0.02 μmole of $AgNO_3$. Protein concentrations were determined by absorbancy at 280 mμ, using an extinction coefficient of 1.25 l./g.

Results

When the pH was raised above pH 12, the γ-globulin molecule immediately swelled, causing a drop in sedimentation coefficient of about 11% and an increase in the viscosity of almost 40% (Table I, Figures 1 and 2). As will be shown later, there was also spectrophotometric evidence indicating that the swelling occurred immediately. Then, with the passage of time at the high pH, a second, more slowly sedimenting peak began to appear. Eventually all of the material was converted into this more slowly sedimenting species. Intermediate species were not seen during this conversion. During this time, the molecular weight dropped by a factor of 0.5, indicating that the molecule had split into half-molecules (Figure 3 and Table I).

The rate at which this breakdown occurred was studied at 0 and also 20°. At 0° the kinetics of breakdown were determined by ultracentrifugation by measuring the relative areas under the two peaks as a function of time at the high pH (Figure 4); at pH 12.0, the rate of breakdown was too slow to be measured by our techniques; at pH 12.2, the half-life was about 100 hr; and at pH 12.4, it was about 30 hr. At the higher temperature, using light-scattering techniques we found the half-life to be only a few minutes at pH 12.4 (Figure 5).

A Study of the Reversibility of the Alkaline Cleavage. A number of attempts have been made to bring about recombination of the fragments formed by alkali
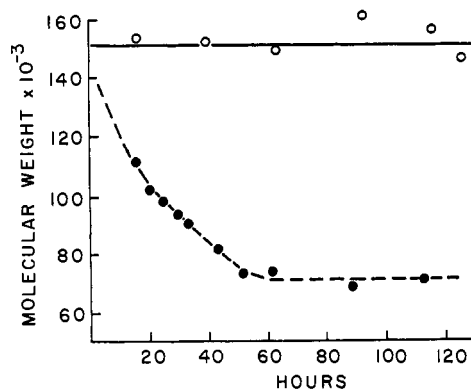

FIGURE 3: Sedimentation equilibrium of γ-globulin at pH 12.5 in 0.15 M KCl–0.015 M lysine (lower curve) and pH 7.0 in 0.15 M KCl–0.04 M iodoacetamide (upper curve). The temperature was 4° and speed was 12,590 rpm.
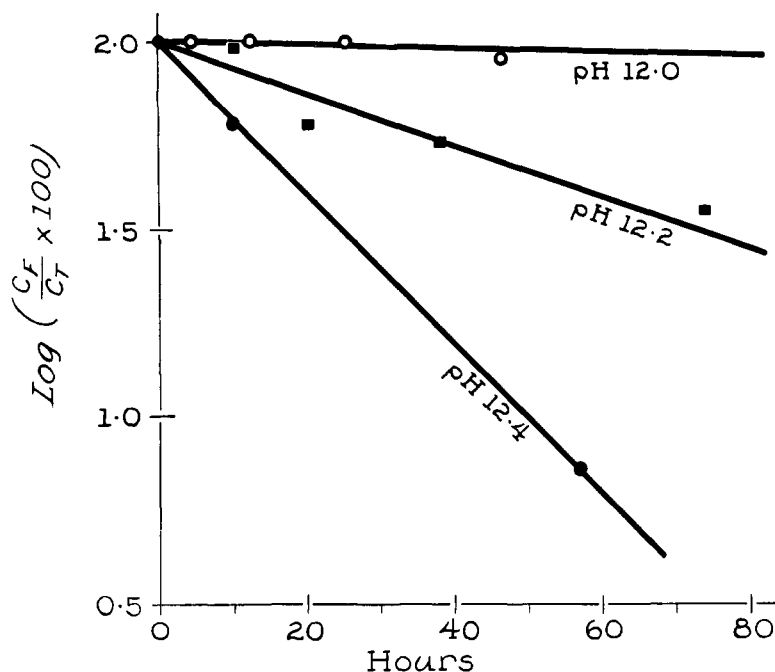
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FIGURE 4: Kinetics of alkali cleavage at various pH values at 0°, plotted as the logarithm of the ratio of the concentration of the faster sedimenting component to the total concentration, as a function of time.

dissociation; however, all such attempts have resulted only in the formation of aggregates. This has been true also for globulin which has undergone prior reduction and alkylation.

The alkali-produced fragments showed antigenic activity when treated with a mixture of goat antilight and antiheavy chain antisera (kindly supplied by Dr. S. Utsumi). With the gel diffusion technique, two precipitin bands were found which fused with those from intact γ-globulin.

As might be expected, both from the apparent distortion of the molecules and from the univalent nature of half-molecules, no precipitate formed between alkali-cleaved antiovalbumin antibody and its antigen. Also, we were unable to demonstrate blocking activity by the dissociation products.

Spectrophotometric Titrations. The basic equation used for the analysis of protein spectrophotometric titration curves may be written as

$$n_s = \frac{n_t k^0(OH^-)e^{+2wZ}10^{+14}}{1 + k^0(OH^-)e^{+2wZ}10^{+14}} \quad (1)$$

where k^0 is the intrinsic H^+ dissociation constant of tyrosine residues on the protein, n_s is the number of ionized tyrosine residues, n_t is the number of unionized residues, (OH^-) is the hydroxyl ion concentration, and Z is the net charge on the protein. The w is the electrostatic interaction parameter, and is inversely related to the size of the protein. When γ-globulin swells above pH 12, w would be expected to drop, and then, after the molecule dissociates, w would

be expected to rise.

The dissociation of the tyrosine residues on the protein was followed by the change which occurs in the ultraviolet absorption spectrum of this group upon dissociation. First, the γ-globulin was titrated from pH 8 to 12.4 and then immediately back-titrated to pH 8 (Figure 6). Since the molecule initially swells at pH 12.4, the value of w should drop, and the back-titration curve would be expected to differ from the forward-titration curve. These data were analyzed by rearranging eq 1 to give

$$\frac{1}{n_s} = \frac{1}{n_t k^0 10^{+14}(OH^-)e^{+2wZ}} + \frac{1}{n_t} \quad (2)$$

Thus, for n_t groups, all of which have an intrinsic pK of $-\log k^0$, a plot of $1/n_s$ vs. $1/(OH^-)e^{+2wZ}$ should give a straight line whose intercept at $1/(OH^-)e^{+2wZ} = 0$ is $1/n_t$ and whose intercept at $1/n_s = 0$ is $-k^0 10^{+14}$. Using the value of $w = 0.013$ for the forward curve and values of Z from Gould et al. (1964) we have drawn such a plot in Figure 7. The corresponding plot for the immediate back-titration curve is straight when a lower value of $w = 0.0099$ is used.

After the γ-globulin had remained at the high pH for a prolonged period, it dissociated into half-molecules, and the values of n_t and Z would be expected to drop by a factor of one-half, while the value of w should increase. In Figure 6 is shown the back-titration curve obtained after 30 hr of pH 12.4, at which time we found by ultracentrifugation that the γ-globulin had completely dissociated. In Figure 7 is shown the
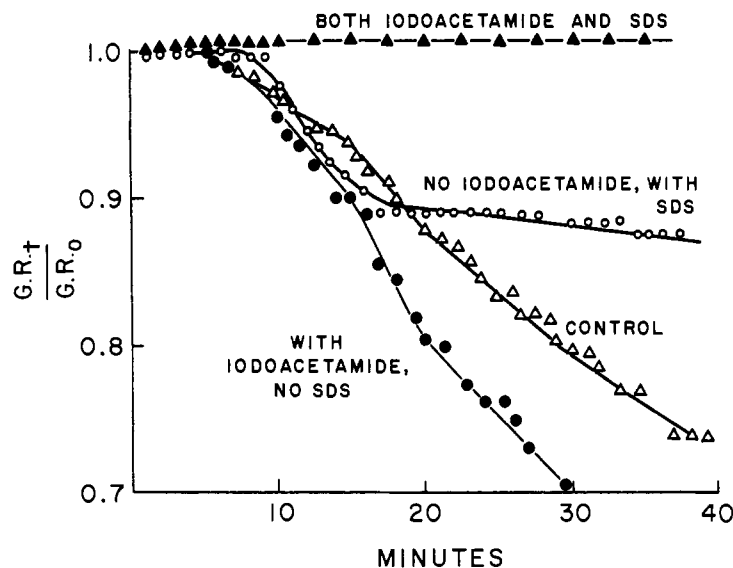
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FIGURE 5: The effect of alkylation in the presence of SDS on dissociation as followed by light-scattering measurements. (upper curve) After preincubation of 2.95 ml of 2% globulin containing 1.5% SDS at pH 8 for 15 min with 0.25 ml of 1 M iodoacetamide, the pH was raised to 12.20 with 1 N KOH. Upon raising the pH, some of the SDS formed micelles and these were removed by filtration through a Millipore filter (0.25 μ), and the scattered light was then measured. (lower curves) Same procedure minus indicated reagents.

corresponding reciprocal plot using values of $Z/2$ and $n_t/2$, and a w value of 0.0125.

In Figure 6, the solid curves represent the experimental data obtained during the spectrophotometric titrations of γ-globulin. The broken curves represent the theoretical titration curves obtained using the best values of n_t and w obtained as a result of these analyses and listed in Table II. These photometric data do not

becomes a sulfenic acid, which then can be further oxidized to a sulfonate, or internally reduced to yield H_2S and an aldehyde. The other sulfur is reduced to SH. Therefore, at least one titratable SH group and perhaps as many as three, should appear for each disulfide bridge hydrolyzed by alkali.

The appearance of new SH groups liberated during

TABLE II: Best Values for the Number of Ionizing Groups the Intrinsic pK, and the Electrostatic Interaction Parameter from Forward and Reverse Spectral Titrations.

Type	n_t	pK^0	w
Forward	32	10.45	0.0133
Reverse (10 min)	32	10.45	0.00899
Reverse (30 hr)	15.9	10.48	0.0125

indicate that hidden tyrosine residues are uncovered, but instead can be entirely explained on the basis of swelling and dissociation of the macromolecules.

Sulfhydryl Groups and Disulfide Bridges. Since the molecules were split in half by alkali treatment, as indicated by the drop in molecular weight, disulfide bonds must have been broken. It is well known that disulfide bonds may be hydrolyzed by alkali (Boyer, 1959). In this cleavage, one of the two sulfur atoms
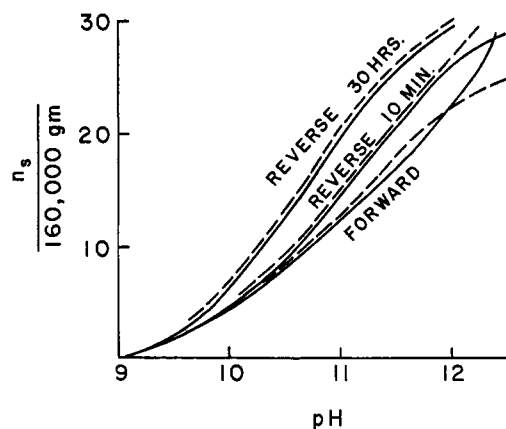

FIGURE 6: Forward and reverse titration curves (————) corrected for turbidity, together with theoretical curves (— — — —). The lower curves are from the forward titration, the middle are from the reverse titrations at 10 min, and the top curves are from the reverse titration at 30 hr. The theoretical curves are computed from the data of Table II. The solvent was 0.15 M KCl–0.015 M lysine plus KOH or HCl.
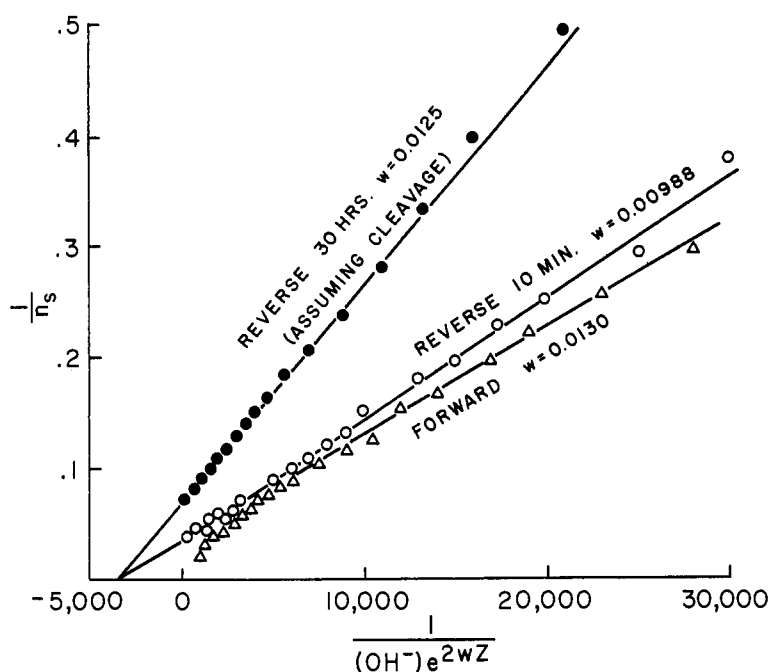
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FIGURE 7: Plots of $1/n_s$ vs. $e/(OH^-)e^{2wZ}$ for the forward and reverse spectrophotometric titrations curves for γ-globulin. Values of n_s were computed by dividing the optical density increment by the difference between the extinction coefficients at 300 mμ for the ionized and unionized tyrosyl residues. Values of Z were taken from the data of Gould *et al.* (1964).

alkali cleavage was followed by the silver ion amperometric titration method using a rotating platinum electrode and a mercuric oxide dropping electrode as described under Materials and Methods. These results are shown in Table III. No SH groups were titratable when native γ-globulin was studied at pH 7.3 unless detergent was first added. But in the presence of detergent, approximately 1.5 SH groups/mole became titratable. This result is in agreement with the work of Karush (1957) and Utsumi and Karush (1964)

and probably indicates that there exists one to two buried SH groups tucked inside the molecule and unavailable for titration unless detergent is added.

Now, as each molecule splits in half, there should appear at least one new sulfhydryl group and perhaps as many as three. So, a series of experiments were performed simultaneously following dissociation by ultracentrifugation and the appearance of new SH groups by amperometric titration. These data are combined in Figure 8, from which it may be seen that as many as 40% of the molecules are cleaved without the appearance of additional SH groups.

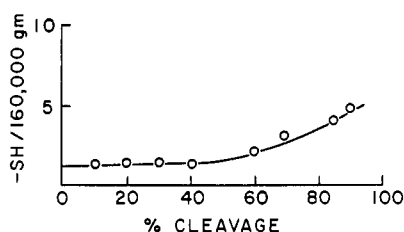At first, this result did not seem to be in agreement

FIGURE 8: A plot of titratable SH groups per mole, determined amperometrically, as a function of the extent of dissociation, measured by ultracentrifugation. The degradation reaction was carried out at 5–7°, under N_2, at pH 12.4. The ultracentrifugation was performed at the same pH at 4° on aliquots removed periodically. The sulfhydryl assays were performed on neutralized aliquots in the presence of 1% SDS.

TABLE III: Sulfhydryl Determination.

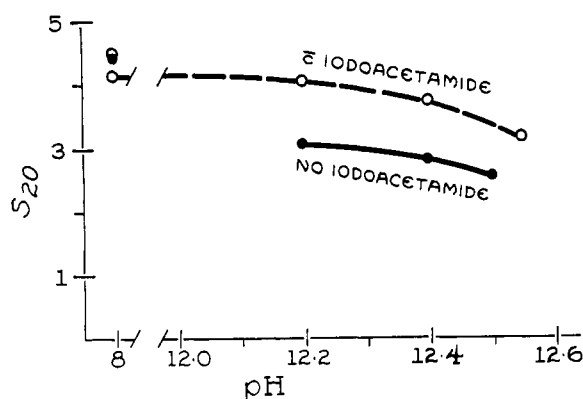| Solution	SH/mole
Solvent (in presence of SDS)	0.0
Reduced glutathione (in presence of SDS)	0.9
Intact γ-globulin (in presence of SDS)	1.4
	1.4
	1.7
	1.5
Intact γ-globulin (in absence of SDS)	0.0

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FIGURE 9: The effect of alkylation of γ-globulin in the presence of detergent on the sedimentation rate of globulin at high pH. Open circles are in the presence and closed circles are in the absence of iodoacetamide. The values are corrected for temperatures, but not for viscosity.
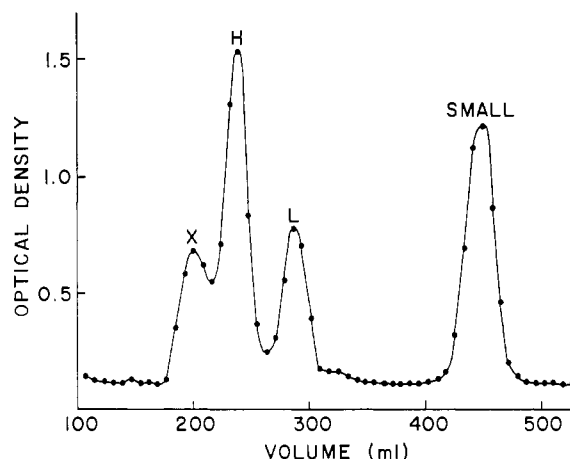

FIGURE 10: Upward-flow chromatography on Sephadex G-200 in 5 M neutralized guanidinium hydrochloride according to the conditions of Small and Lamm (1966). γ-Globulin exposed to alkali at pH 12.4 for 2 hr at 25° was shown to dissociate entirely into 3S components. These were reduced and alkylated in 5 M guanidinium hydrochloride and applied to the column. The aggregated material (X) has a molecular weight of 200,000. The heavy and light chains have molecular weights of 55,000 and 27,000, respectively. The trailing peak, appearing at the end of the entire column volume, represents small molecules such as iodoacetamide present in the original incubation mixture.

with the excellent evidence for the existence of at least one disulfide bridge between the two halves. There is, however, another mechanism by which the molecule might be split into half-molecules without the appearance of additional sulfhydryl groups. At pH values above 12, the molecule swells, and it is possible that the buried sulfhydryl is brought next to the disulfide bridge. Catalyzed by alkali, a disulfide-exchange reaction might then occur. This would cause a cleavage of the molecule to yield half-molecules. There would be no increase in the total number of sulfhydryl groups.

This hypothesis was tested. By alkylation of the buried SH group prior to the alkali treatment, it should be possible to prevent the exchange reaction. The results of such experiments are shown in 5, in which the molecular weight changes were followed by light scattering.

The alkylation reaction was performed in the presence of detergent to expose the buried sulfhydryl. In the absence of detergent, neither iodoacetic acid nor iodoacetamide prevented breakdown. In the presence of detergent only, breakdown proceeded slowly. But preincubation both with detergent and iodoacetamide markedly inhibited breakdown, suggesting that an exchange reaction was required for dissociation.

In addition, sedimentation of globulin which had been preincubated with iodoacetamide in the presence of detergent and then exposed to pH > 12.0 showed a peak which was sharper and sedimented at least 30% more rapidly than its control which had been at the same pH and at the same temperature, but which lacked the alkylating agent. These data are summarized in Figure 9. It was also found that the presence of 0.1 M mercaptoethanolamine increased the rate of dissociation at pH 12.0, and that γ-globulin which

was first reduced and alkylated dissociated rapidly at pH 11.5.

Preparation of Heavy and Light Chains after Alkali Dissociation. In order to show that alkali does not cause destruction of the heavy and light chains at the pH values employed in these experiments, γG-immunoglobulin was treated at pH 12.4 for 2 hr at 25°. Examination in the centrifuge showed that all the material had been converted to the 3S species. The material was then dissolved in guanidine hydrochloride at pH 7, reduced with mercaptoethanol, and alkylated according to the procedure of Small and Lamm (1966).

Chains were then separated by upward-flow chromatography on Sephadex G-200 in 5.5 M neutralized guanidine hydrochloride. The amount of material placed on the column was 94.42 and 78.4 mg or 83.4% was recovered. Of the recovered material, 12.8 mg was an aggregate which came off as a leading peak at the end of the void volume (Figure 10). The amount of heavy chains recovered (42.1 mg) was roughly twice that of light chains (23.5 mg).

Molecular weights were measured at low and high speeds at three different protein concentrations and values extrapolated to infinite dilution. For the aggregate, the heavy, and the light chains, respectively, values of 200,000, 55,000, and 27,000 were obtained. The presence of aggregate has been reported by other workers, and is thought to

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be a mixture of heavy and light chains with a molecular weight of about 180,000 (Lamm and Small, 1966).

Discussion

As the pH of a solution of γ-globulin is increased, a swelling of the molecule occurs at a pH of about 12.0. Evidence for this is supplied both in the observed increase in intrinsic viscosity and decrease in the sedimentation coefficient, and also from the observation that the spectral titration curve deviates from a theoretical curve at this pH, and gives, upon immediate back-titration, a lower value for w. The latter finding also indicates that this swelling is not reversible, since this lower value of w was obtained over a pH range of 9.5–12.4.

We suggest that this swelling favors a disulfide-sulfhydryl-interchange reaction which effectively eliminates the interheavy–heavy chain disulfide bond, and allows the molecule to dissociate into halves. Evidence for an interchange mechanism is the lag in appearance of additional sulfhydryl groups. This interchange appears to require the participation of one or more buried sulfhydryl groups, since alkylation in the presence of SDS[1] inhibits the dissociation. That this sulfhydryl is "buried" is suggested by the observation that exposure to the alkylating reagent in the absence of detergent does not inhibit the dissociation at room temperature. This sulfhydryl is probably the same one which is not titratable with silver ion in the absence of detergent, but becomes titratable in the presence of 1% SDS.

That cleavage of the molecule by alkali does occur is supported by hydrodynamic, light-scattering, and spectral data. The actual cleavage appears to have an initial lag phase, when studied by light scattering at room temperature, but obeys first-order kinetics at low temperature. In the latter experiments, the lag phase may have been too short to be detected by serial sedimentation analysis. In any event, the rate of cleavage is strongly dependent upon the pH of the solution. This may be due to the fact that the degree of swelling is highly pH dependent, and thus the probability that the sulfhydryl and disulfide groups will be in the proper configuration for interchange to take place. Dissociation will occur at a lower pH in the presence of reducing agents, or after reduction and alkylation, and these facts are also consistent with the present hypothesis. Such treatment would eliminate the requirement for an interchange reaction involving the "buried" sulfhydryl group. The high pH is then required only to disrupt the noncovalent forces, rather than to alter the rate of the interchange reaction.

Products of the cleavage appear to be half-molecules, at least initially, since their molecular weight by velocity and equilibrium sedimentation analysis is about 70,000 and since such an assumption is consistent with the

spectral titration data. According to the latter, no additional tyrosine groups are exposed at pH values up to 12.4, and there is no change in their intrinsic pK values.

The interaction parameter, w, increases over its value for the irreversibly swollen, nondissociated molecule, again indicating cleavage.

The distortion of conformation which takes place as soon as pH > 12.0 is reached, is apparently sufficient that the half-molecules are unable to recombine in a specific manner, but instead form aggregates when the pH is lowered.

As would be expected, both from the distortion and also from the fact that the halves would contain only single combining sites, the products derived from antibody γ-globulin lose their ability to form precipitate with antigen. We were also unable to demonstrate "blocking" activity. As the distorted molecules continue to be exposed to alkali, a gradual hydrolysis of disulfide occurs, and thus an increase in titratable sulfhydryl is seen.

Intact globulin has no titratable sulfhydryl groups in the absence of detergent; however, about 1.5 sulfhydryl groups appear in 1% SDS. The detergent also causes the sedimentation rate of intact globulin to decrease to about 4.5 S. At neutral pH, the sedimentation rate of a 1% solution of reduced and alkylated globulin also decreases with increasing SDS concentration until it reaches a plateau value at about 1% SDS and remains at 3.8–4.0 S with further increase in SDS concentration.

On the other hand, there is no apparent change in the sedimentation rate of the 3S products of pepsin digestion, reduction, and alkylation with detergent treatment.

We believe that the effect of SDS on γ-globulin can be explained by an unfolding of the molecule, resulting in an increase in axial ratio. This unfolding evidently brings a free sulfhydryl group to a position where it is accessible to silver ion and alkylating agents.

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1436   [1] Abbreviation used: SDS, sodium dodecyl sulfate.

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Fluorescence Polarization of Human γG-Immunoglobulins*

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ABSTRACT: The fluorescence polarization of 1-dimethyl-aminonaphthalene-5-sulfonyl conjugates of human γG-immunoglobulins (DNS-IgG) has been measured under various conditions. Rotational relaxation times at 25° were calculated from experiments in which the viscosity of the solvent was varied by altering the temperature. Different rotational relaxation times were obtained when the solvent viscosity was altered by addition of sucrose at fixed temperatures. The results are explained by assuming that DNS groups covalently bound to the immunoglobulin undergo thermally activated rotations independent of the rotation of the macromolecule. This hypothesis was tested by experiments which showed that addition of a DNS conjugate of ε-aminocaproic acid to solutions of DNS conjugates of immunoglobulin mimics the effect of temperature on the immunoglobulin conjugates in the absence of any fluorescent small molecules. Moreover, on the basis of this hypothesis, the polarizations in heated solutions were calculated from polarizations of DNS-IgG measured in sucrose solutions at fixed temperatures (sucrose isotherms). A rotational relaxation time of approximately 221 nsec was obtained both for human γG-immunoglobulin and human γG-myeloma protein. This value is in agreement with reported estimates obtained by other relaxation methods and is considerably higher than values previously obtained from fluorescence polarization measurements. An effect of the wavelength of excitation on the polarization of fluorescence was also noted. This effect appeared to be related to the presence of different environments of covalently bound DNS groups. The present findings are consistent with rodlike models of the IgG and incompatible with extreme molecular flexibility.

The fluorescence polarization of 1-dimethyl-aminonaphthalene-5-sulfonyl conjugates[1] of γG-immunoglobulins has been studied in a number of laboratories (Steiner and Edelhoch, 1962; Chowdhury and Johnson, 1963; Winkler, 1965). The results of such studies have been used to compute a rotational relaxation time (ρ_h) of the macromolecule. In general, the reported values of ρ_h (approximately 100 nsec) are lower than would be expected if the γG-immunoglobulin molecule behaved as a rod-shaped particle (Edelman and Gally, 1964) with dimensions of 240 × 57 × 19 A (Kratky *et al.*, 1955). This discrepancy has usually been interpreted as an indication that the DNS group is attached to macromolecular regions that are capable of independent rotation. On these and other grounds, it has been proposed that the γG-immunoglobulin molecule is a flexible structure (Noelken *et al.*, 1965).

An alternative explanation for the observed values of ρ_h is that covalently bound DNS groups undergo thermally activated rotations independent of macromolecular rotations (Weber and Teale, 1965). In the present study, we provide evidence that thermally activated rotation of conjugated DNS groups is largely responsible for lowering the ρ_h values of γG-immunoglobulins. Using a theory which relates fluorescence polarization data obtained at different temperatures and viscosities (Weber, 1952a), the ρ_h of immunoglobulin is estimated to be approximately 221 nsec. This value is consistent with the dimensions and shape parameters calculated from data obtained by a variety of methods (Kratky *et al.*, 1955; Noelken *et al.*, 1965). Although γG-immunoglobulin molecules may have some degree of flexibility, the fluorescence polarization of DNS conjugates does not provide compelling evidence that it exists.

Materials and Methods

All chemicals were reagent grade unless otherwise

[1] Abbreviations used: DNS, 1-dimethylaminonaphthalene-5-sulfonyl; ρ_h, rotational relaxation time. Unless otherwise indicated, ρ_h was calculated for a temperature of 25°. EACA, ε-aminocaproic acid; IgG, γG-immunoglobulin; Eu, γG-myeloma protein.

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