ON THE MODIFICATION OF γ-GLOBULIN BY ACID*

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INTRODUCTION

It has recently been shown[1] that in acidic media the sedimentation behavior of conalbumin exhibits a complex type of variation with changes in either pH or ionic strength of the solution. Viscosity and osmotic pressure measurements, along with sedimentation analyses, led to the conclusion that, under certain conditions (*e.g.*, ionic strength 0.1; pH changing from 5.3 to 3.0), the protein undergoes configurational changes which affect its sedimentation constant, but are without influence on its molecular weight. With properly chosen salt concentration, however, acid pH's also can cause aggregation. The purpose of the present communication is to show that, on exposure to acidic media, bovine γ-pseudoglobulin behaves in a manner similar to conalbumin. In both instances the configurational changes can be interpreted in terms of molecular swelling. The chief differences seem to be that, whereas conalbumin appears to undergo an essentially isotropic swelling, γ-globulin seems to swell anisotropically.

EXPERIMENTAL

The physicochemical methods used in this study have been described in detail previously[1]. Experiments were carried out in NaCl and NaCl–HCl solutions. Sedimentation constants have been corrected to water at 20° C as hypothetical solvent and are reported in Svedberg units ($S = 1 \cdot 10^{-13}$ sec^{-1}).

The bovine γ-pseudoglobulin used in most of these experiments was the water-soluble fraction of γ-globulin prepared by the method of electrophoresis-convection[2]. The globulin fraction precipitated from fresh bovine serum by the addition of one volume of saturated ammonium sulfate, was fractionated by a single 48 hour stage of electrophoresis-convection at pH 7.0 in ionic strength 0.1 phosphate buffer. Portions of the resulting top fraction (γ-globulin) were dialyzed exhaustively against many changes of cold, distilled water; the water-insoluble fractions removed by centrifugation and discarded, and the water-soluble fractions stored in salt-free solution at 2° C. Both the whole γ-globulin and the pseudoglobulin migrated as a single boundary in an electric field at pH 8.6, ionic strength 0.1 barbital buffer; and about 98–99% of the globulin sedimented as a single boundary in the ultracentrifuge.

Some of the sedimentation experiments (particularly those shown in Figs. 1 and 2) were carried out on γ-pseudoglobulin prepared from Armour Fraction II of Bovine Plasma. The extrapolated sedimentation constants of the two different preparations were the same, but the intrinsic viscosity of the pseudoglobulin from Fraction II was about 15% greater than that of pseudoglobulin from the material prepared by electrophoresis-convection. Other differences between the two materials were: (a) at pH 7 the pseudoglobulin from Fraction II contained

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10–15% of a 10 *S* component, the relative amount of this component decreasing as the total protein concentration was lowered and as the pH was changed from 7 to 3; and (b) the slope of the plot of sedimentation constant *vs.* concentration of the Armour material was twice as great as that of the material prepared by electrophoresis-convection.

RESULTS

Sedimentation studies

Bovine γ-pseudoglobulin was examined in the ultracentrifuge at pH values ranging from 7.4 to 2.0 and at ionic strength 0.1. The corrected sedimentation constant of the major portion of γ-globulin* is plotted against pH in Fig. 1. These data show that, whereas γ-globulin sediments at a rate independent of pH over the range 7.4 to 4.2, the sedimentation contant of the protein decreases by about 10% between pH 4.2 and 3.4 and then once again becomes independent of pH over the range 3.4 to 2.0.

In nearly neutral solutions the sedimentation behavior of γ-pseudoglobulin is independent of salt concentration over the range 0.02 to 1 *M* NaCl, but, as shown in Fig. 2, the sedimentation behavior is strongly dependent on salt concentration at pH 3.1. In acidic solutions of relatively low salt concentrations the protein sediments in most instances as a single boundary** at a rate which decreases continuously from

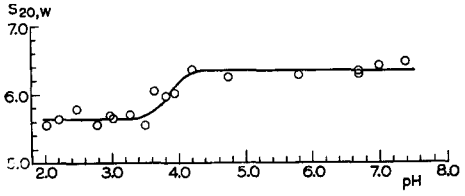

Fig. 1. Effect of pH on the sedimentation constant of bovine γ-pseudoglobulin at ionic strength 0.1; protein concentration about 1.3 g/100 ml.

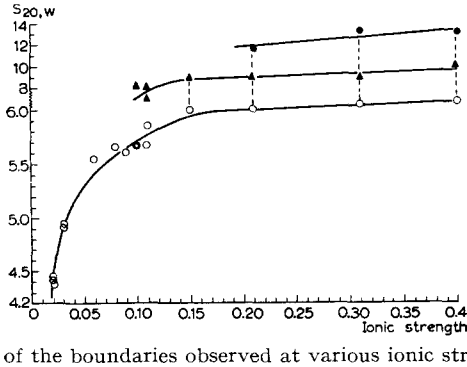

Fig. 2. Effect of ionic strength on the sedimentation behavior of bovine γ-pseudoglobulin at pH 3.1, protein concentration about 1.3 g/100 ml. The plot shows the sedimentation constants of the boundaries observed at various ionic strengths. At ionic strengths 0.21–0.40, for example, three sedimenting boundaries were observed; at ionic strengths 0.10–0.15, two boundaries, etc. Only a few per cent of the faster moving boundary was observed at ionic strength 0.1.

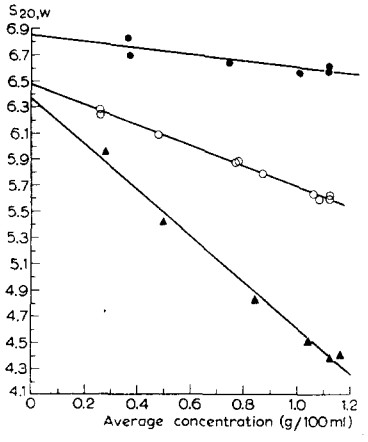

Fig. 3. Dependence of the sedimentation constant of bovine γ-pseudoglobulin on protein concentration: ●, pH 6.5–7 ionic strength 0.10; ○, pH 3.1 ionic strength 0.1; ▲, pH 3.1 ionic strength 0.02.

* At pH 3 γ-globulin contains only a few percent of a 7–8.5 *S* component, but when the pH is lowered further the proportion of this component increases and new, more rapidly sedimenting boundaries appear. For example, at pH 2, 20% of the protein sediments with sedimentation constants of 8.1 and 10 *S* and 5% with a sedimentation constant greater than 10 *S*.

** At the lowest salt concentration the sedimenting boundary was skewed in a peculiar manner suggesting heterogeneity.

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5.7 *S* to 4.4 *S* as the ionic strength is varied from 0.1 to 0.02, respectively*. At higher salt concentrations, the sedimentation patterns show three boundaries; at ionic strength 0.3, for example, the protein contained 82% of a 6.23 *S* component; 13%, 9.5 *S*; and 5%, 12 *S*. The appearance of the more rapidly sedimenting components at high salt concentrations has been attributed to aggregation of the protein[4].

Plots of the corrected sedimentation constants under different conditions of pH and ionic strength against the average concentration of the solution through which the protein sedimented, are shown in Fig. 3. Extrapolation of such plots to infinite dilution yields sedimentation constants characteristic of the protein and independent of concentration effects. Plots of the reciprocal of the sedimentation constant *vs.* average concentration were also linear. The extrapolated sedimentation constants obtained from the two types of plots were in excellent agreement except for the case of pH 3.1 and ionic strength 0.02. In that case the extrapolated constant was about 4% higher when obtained from $1/s$ *vs.* c than s *vs.* c. The least squared lines of regression relating sedimentation constant to protein concentration are given in Table I. It will be seen that at ionic strength 0.1, both the extrapolated sedimentation constant and the slope of s *vs.* c are sensitive to pH. However these data show that, whereas the sedimentation constant of a 1.3% solution of γ-globulin (average concentration about 1.1%) at pH 3.1 decreases by about 20% on going from ionic strength 0.1 to 0.02, the extrapolated sedimentation constant does not decrease and may even increase.

TABLE I

DEPENDENCE OF SEDIMENTATION CONSTANT ON PROTEIN CONCENTRATION

pH and salt concentration	*Equation of line* of regression*
pH 6.5–7.0, $\Gamma/2$ 0.1	$s = 6.85 - 0.265c$ $1/s = 0.146 + 0.0059c$
pH 3.1, $\Gamma/2$ 0.1	$s = 6.47 - 0.797c$ $1/s = 0.154 + 0.0228c$
pH 3.1, $\Gamma/2$ 0.02	$s = 6.37 - 1.77c$ $1/s = 0.149 + 0.0690c$

* The symbol, s, represents the corrected sedimentation constant, and c, the average protein concentration, g/100 ml.

The effect of pH on the sedimentation behavior of bovine γ-pseudoglobulin is not completely reversible, aggregation occurring on neutralization of acidic solutions**. For example, after a 1 hour exposure to pH 3.1, ionic strength 0.1, followed by dialysis*** first against phosphate buffer at pH 7 and then 0.1 *M* NaCl, the protein solution

* That the protein sediments with the same sedimentation constant in KCl–HCl solutions at pH 3.1, salt concentrations 0.1 and 0.02 *M* as in NaCl–HCl at the same pH and salt concentrations, indicates that the dependence of the sedimentation constant on salt concentrations, is not due to a primary charge effect[3]. The primary charge effect would be expected to be minimal in solutions of KCl (personal communication from Professor JOHN G. KIRKWOOD).

** γ-Globulin from rabbit anti-phage T2 serum[5] showed a reversible change in sedimentation constant on going from pH 6.5 to 3. Its phage neutralizing activity was not affected by exposure to pH 3 and there was no loss in specificity.

*** In one case some precipitation occurred during dialysis. When aliquots of protein solutions at pH 2.8–2.0 were added to phosphate buffer at pH 7.5, considerable precipitation always occurred.

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had a bluish hue and now yielded a precipitate when dialyzed against distilled water. The "reneutralized" material contained 64% of a 6.69 *S* component; 8%, 9 *S*; 11%, 13 *S*; 7%, 24 *S*; 10%, 66 *S*. Its water-soluble fraction contained 93%, 6.51 *S* and 7%, 11 *S*; and the extrapolated sedimentation constant of the major component was the same as that of native protein. In contrast, the water-insoluble fraction when redissolved in 0.1 *M* NaCl and clarified, was bluish and contained 26%, 6.7 *S* and 74%, 23 *S* (approximately one-half of the water-insoluble fraction redissolved in 0.1 *M* NaCl).

Viscosity measurements

The results of the viscosity measurements are presented in Fig. 4, where the reduced specific viscosity, η_{sp}/c, is plotted against the protein concentration, c, in g/100 ml. Intrinsic viscosities, $[\eta]$, were obtained by extrapolation of least square lines of regression to infinite dilution and are given in the legend to the figure. These data show that the intrinsic viscosity of γ-pseudoglobulin is insensitive to pH at ionic strength 0.1–0.15, but that the viscosity at pH 3.1 increases about 60% when the ionic strength is lowered from 0.1 to 0.02. By reasoning similar to that previously used, footnote 7 of reference[1b], we interpret this change in viscosity as a reflection of structural changes rather than an electroviscous effect.

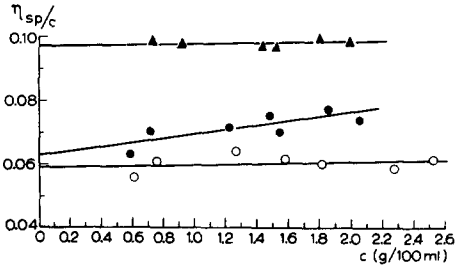

Fig. 4. Dependence of reduced specific viscosity, η_{sp}/c, on protein concentration, c. ○, pH 6–7 ionic strength 0.15, $[\eta] = 0.059$; ●, pH 3.1 ionic strength 0.1, $[\eta] = 0.063$; ▲, pH 3.1 ionic strength 0.02, $[\eta] = 0.097$.

Osmotic pressure measurements.

The osmotic pressures, π, of γ-pseudoglobulin solutions of protein concentrations, c, ranging from about 3.2–2 to 0.4 g/100 ml, have been measured at pH 5.3–7 and at pH 3.1, ionic strength 0.1. The number average molecular weights were determined by extrapolation of π/c *vs.* c to infinite dilution*. The molecular weight of the native pseudoglobulin** was 147,000. In the case of two preparations, the molecular weight*** did not change on exposure to pH 3.1; while the molecular weight of a third preparation decreased to 119,000 indicating that some molecular dissociation into smaller units had occurred. (This preparation was not used for sedimentation or viscosity measurements.)

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* With one preparation at pH 7 the plot was linear and the slope negative, while with a second preparation at pH 5.3 the plot was non-linear and concave downward. At pH 3.1 all the plots were linear and had positive slopes.

** A preparation of γ-globulin never exposed to distilled water had a molecular weight of 169,000 at pH 5.3 and 147,000 at pH 3.1. Whole γ-globulin may contain aggregates which dissociate at acid pH's and are insoluble in distilled water.

*** At pH 3.1 the sedimentation patterns of pseudoglobulin prepared from Armour's Fraction II were the same as those of the water-soluble fraction of γ-globulin prepared by electrophoresis-convection, and its molecular weight at this pH and ionic strength 0.1 was also the same, 151,000 ± 8,900 (10 determinations). The molecular weight at ionic strength 0.02 was 172,000 ± 17,400 (7 determinations) but the difference between the molecular weight at the two ionic strengths is not statistically significant.

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DISCUSSION

The decrease in sedimentation constant of γ-pseudoglobulin on going from pH 4.2 to 3.4 (ionic strength 0.1), unaccompanied by changes in intrinsic viscosity and, in most instances, molecular weight, suggests that the γ-globulin molecule swells anisotropically on exposure to mildly acidic media, *i.e.*, the volume of the molecule increases and at the same time its shape becomes more symmetrical. This conclusion is supported by calculations of possible molecular dimensions for γ-globulin at pH 6–7 and 3.1. The dimensions of a spheroidal hydrodynamic model were estimated using the three most reliable of the methods* recently considered by OGSTON[6]. The results of these computations are presented in Table II, where β is a function of the ellipicity J of the spheroidal model, *i.e.*, the ratio of the semi-axis of revolution to the equatorial semi-axis; V', the effective hydrodynamic volume per g of unhydrated mass; and M, the unhydrated mass, *i.e.*, molecular weight. Although the agreement between the three methods leaves something to be desired, all three indicate that the axial ratio of the γ-globulin molecule decreases considerably on going from pH 6–7 to 3.1 and that this change in shape is accompanied by a large increase in molecular volume, presumably due to imbibation of solvent. It would seem that this configurational change, which occurs over a very narrow pH range, as shown in Fig. 1, is a cooperative or "all-or-none" phenomenon, perhaps resulting from breakage of a critical number of intramolecular bonds, possibly hydrogen bonds. That an additional, continuous change in structure occurs is indicated by the appearance of more rapidly sedimenting components** in solution more acidic than pH 3.

TABLE II

DIMENSIONS OF γ-PSEUDOGLOBULIN MOLECULE CALCULATED FROM EXPERIMENTAL DATA*

Solvent	*Method I*			*Method IIa* ($\varkappa = 3$)			*Method IIb* ($\varkappa = 1.8$)		
	β	J	V'	M	J	V'	M	J	V'
pH 6–7, $\Gamma/2$ 0.10–0.15	2.20	4	1.3	$1.2 \cdot 10^5$	17	0.20	$1.3 \cdot 10^5$	11	0.44
pH 3.1, $\Gamma/2$ 0.1	2.12	1–1/2	2.5–2.2	$1.4 \cdot 10^5$	4.8	1.1	$1.5 \cdot 10^5$	1/4.5	1.5

* Measured partial specific volume of 0.735 was used in the calculations. This is somewhat larger than the values of 0.720 and 0.725 reported for "whole" bovine γ-globulin[8].

Interpretation of the changes in the hydrodynamic properties of γ-globulin observed on going from ionic strength 0.1 to 0.02, pH 3.1, may be complicated by aggregation***. It would seem, however, that further changes in molecular configuration occur on lowering the salt concentration, possibly due to increased coulombic repulsion

* These methods make use of two independent hydrodynamic measurements without making assumptions regarding hydration. Method I, first formulated by SCHERAGA AND MANDELKERN[7], makes use of the extrapolated sedimentation constant $s^{\infty}_{20,w}$, intrinsic viscosity, partial specific volume and unhydrated mass. The values of axial ratios calculated by this method are extremely sensitive to errors in experimental data, particularly in the partial specific volume. Method II circumvents this difficulty by making use of $s^{\infty}_{20,w}$, $d(1/s)/dc$ and $[\eta]$, but involves a parameter, $\varkappa$, whose value is uncertain. OGSTON[6] uses the values, 3 and 1.8, Method IIa and IIb, respectively. The molecular weight can also be estimated by Method II without a knowledge of the diffusion constant, but for this calculation the partial specific volume is required.

** See first footnote page 150.

*** See third footnote page 152.

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within the molecule. If it is assumed that no change in molecular weight occurs, then the data suggest that, in contrast to the effect of pH on configuration, lowering the salt concentration of acidic solutions results in a large and continuous increase in molecular asymmetry, accompanied by a decrease in molecular volume.

As a result of the structural changes incurred on exposure to acidic media, similarly charged globulin molecules can aggregate in these media. At pH 3.1 aggregation apparently occurs, possibly through the formation of intermolecular hydrogen-bonds, when the large positive charges carried by the protein molecules at this pH are shielded sufficiently by means of a double layer to allow close approach. The extent of aggregation depends upon the anionic nature of the supporting electrolyte. This has recently been attributed[4] to reduction of the net charge on the protein molecules as a result of anion binding.

At least some of the configurational changes incurred on exposure to acid are reversible. However, in the case of bovine γ-globulin, aggregation occurs when solutions at pH 3.1 are readjusted to nearly neutral reaction. This is understandable if formation of intermolecular bonds, possibly hydrogen bonds, between molecules of low net charge, competes with reformation of intramolecular bonds when the pH is raised.

It is of interest to note that the concept of molecular swelling, rather than unfolding, has now been evoked to describe changes in the properties of several proteins on exposure to denaturing conditions: the acid modification of conalbumin[1], γ-globulin and serum albumin[9], and the urea denaturation of horse and bovine serum albumin[7]. If a globular protein can be described in terms of a bundle of rods or a polypeptide chain, perhaps with an interrupted helical structure folded on itself, then swelling might be understood in terms of changes in spacing and/or relative orientation of the rods or folds without changes in the rods themselves.

SUMMARY

Bovine γ-pseudoglobulin undergoes structural modification on exposure to media more acidic than pH 4.2. By the concomitant use of hydrodynamic and osmotic pressure measurements the conclusion is reached that under certain conditions (*e.g.*, ionic strength 0.1, pH changing from 7 to 3.1) the protein undergoes configurational changes which affect its sedimentation constant, but are usually without influence on its molecular weight. With properly chosen salt concentration, however, acid pH's also can cause aggregation of the protein molecules.

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