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Conformation Changes and Dissociation of Fc Fragments of Rabbit Immunoglobulin G as a Function of pH

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1. The sedimentation coefficients of rabbit immunoglobulin G, four types of Fc fragments, univalent Fab and bivalent $F(ab)_2$ fragments were measured as a function of pH. 2. In conjunction with molecular-weight determinations by sedimentation equilibrium, and with the behaviour on gel filtration, this enabled the state of association of the Fc fragments to be followed. 3. The type possessing an interchain disulphide bond, lFc fragment, changed extensively in structure, but not in molecular weight. 4. There was good correlation between the readiness to dissociate and the chain length of the shorter Fc fragments that do not contain the interchain covalent bond. 5. The increasing resistance to dissociation as the fragments became shorter ran parallel with the ability to resist enzymic attack. 6. The site of the strong association between component chains of Fc fragment is located in the *C*-terminal half. 7. The gel-filtration behaviour of the Fc fragments clearly confirms that the process is governed by the Stokes radius rather than molecular weight. 8. The ultracentrifugal results were used to estimate the separations of the hydrodynamic subunits in intact immunoglobulin G, and as a basis for a schematic structure.

Non-covalent interactions as well as disulphide bridges play an important role in maintaining the integrity of the rabbit IgG† molecule (Edelman & Poulik, 1961; Fleischman, Pain & Porter, 1962). Dissociation of the two heavy chains is effected by merely lowering the pH to 2·4, provided that the interchain disulphide bridge has been cleaved (Palmer, Nisonoff & Van Holde, 1963). The heavy–heavy-chain noncovalent interactions are located almost exclusively in the Fc region, the *C*-terminal halves of the two heavy chains (Inman & Nisonoff, 1966*a*,*b*); the other halves of the chains are engaged mainly in association with light chains in the Fab region. A schematic arrangement of the polypeptide chains in the IgG molecule has been given by Porter (1962). (This nomenclature of the fragments follows the recommendations of the World Health Organisation, 1964.)

In the preceding paper (Utsumi, 1969), four types of Fc fragments, all products of papain digestion of rabbit IgG, were differentiated and chemically characterized. They are lFc fragment, with a single interchain disulphide bridge at its *N*-terminal end, and mFc, sFc, and stFc fragments, having approximately 220, 210, 170 and 110 amino acid residues/chain respectively. All the fragments contain the *C*-terminal portion of the molecule intact. In the present study, progressive changes in the conformation of Fc fragment, as a function of pH, were investigated by ultracentrifugal and gel-filtration studies of these fragments. Information about the gross physical structure of IgG was obtained by combining the results with those given by monomeric Fab and dimeric $F(ab)_2$ fragments.

MATERIALS AND METHODS

Preparation of rabbit IgG. Rabbit IgG was prepared from pooled rabbit serum as described in the preceding paper (Utsumi, 1969).

Preparation of Fc fragments. Four types of Fc fragments with different polypeptide chain lengths were prepared by the methods described in the preceding paper (Utsumi, 1969). Fragment lFc was isolated by crystallization from the mildly reduced pre-split molecule, a product of brief treatment of IgG with papain (Koch–Light Laboratories Ltd., Colnbrook, Bucks.). By the time of the final crystallization of lFc fragment, over 90% of the molecules had interchain disulphide bridges, as judged by non-dissociability at pH 2·35. Crude fragment mFc from a prolonged digestion of IgG at pH 7·5 in the presence of reducing agent (Procedure I), was purified by elution from

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†Abbreviation: IgG, immunoglobulin G.

DEAE-cellulose (W. and R. Balston, Maidstone, Kent) with 8mM-tris–HCl buffer, pH 8·0. Fragment sFc was obtained by DEAE-cellulose chromatography of a product of papain digestion at pH 5·0, as described by Utsumi (1969). For fragment stFc, the peptic fragment Pep-III′ (Utsumi & Karush, 1965) was used.

Preparation of Fab fragment. Crude Fab fragment, the univalent fragment, obtained as the supernatant of the crystallization of lFc fragment above, was further treated with pepsin (twice crystallized; Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) for 24hr. at pH 4·5 and 37° in 0·1M-sodium acetate buffer. The digest was fractionated by gel filtration on Sephadex G-75 at neutrality. The 3s fraction contained only Fab fragment.

Preparation of bivalent fragment $F(ab)_2$ by tryptic digestion of IgG. A solution of IgG (2·5%, w/v) in 0·1M-NaCl was adjusted to pH 2·3–2·4 with HCl, and mixed with trypsin (twice crystallized; Worthington Biochemical Corp., Freehold, N.J., U.S.A.), which had been dissolved in 10mM-HCl at a protein concentration of 1%. The enzyme/IgG ratio was 1:50 (w/w). Digestion was for 24hr. at 37°, the pH being maintained between 2·2 and 2·4 by addition of 10mM-HCl. The pH was then adjusted to 7·5 with 2M-tris, and the ensuing cloudiness was cleared by repeated freezing (in a solid CO_2–acetone bath) and thawing. The digest was placed on a column of Sephadex G-150 (Pharmacia, Uppsala, Sweden) in 20mM-sodium phosphate–0·2M NaCl buffer, pH 7·8, containing 2mM-EDTA and the 5s fraction was isolated. It contained small amounts of material of approx. 3s and 7s. The fraction was antigenically identical with the papain Fab fragment, and was converted into 3s components if reduced with 14mM-mercaptoethanol.

Preparation of solvents and protein solutions. To minimize changes of environment, apart from pH, the solvent was usually 0·1M-NaCl containing a low-capacity buffer. Between pH 3·5 and pH 5·5 this was 10mM-acetic acid, adjusted with NaOH. Below pH 3·5, HCl alone was added to the NaCl, whereas above pH 5·5 the solvent contained 10mM-tris adjusted with HCl. Protein solutions were generally dialysed against the solvent and concentrations determined in a differential refractometer (Cecil & Ogston, 1951).

Sedimentation-velocity experiments. These were done at 59780rev./min. and approximately 20° in a Spinco model E ultracentrifuge equipped with a temperature-control unit. Records were obtained by schlieren optics, which included a phase-plate and a Wratten 77A filter to isolate light of wavelength 546mμ. The cells were either 3mm. or 12mm., Kel-F centrepieces being used below pH 5. Synthetic-boundary cells were used for samples of low protein concentration or low sedimentation cofficient to improve accuracy. All sedimentation coefficients were corrected to water at 20° in the usual way and expressed in Svedberg units. In the calculations $\bar{v}$ was taken as 0·738 (Noelken, Nelson, Buckley & Tanford, 1965). Viscosity and density determinations were made on tris buffers by standard methods. At each pH, the sedimentation coefficient of each protein was measured at three (in a few cases, two) different concentrations so that an estimate of $S^0_{20,w}$ could be made.

Molecular-weight measurements. These were done at 25° by using either the short-column multichannel cell (Yphantis, 1960) and schlieren optics or, where more detailed information was required, the newer equilibrium techniques developed with interference optics (Charlwood, 1965, 1967*a*,*b*, 1968). In both cases it was assumed that refractive increment was proportional to protein concentration.

When the multichannel cell was used, a series of five photographs was taken at 16min. intervals after equilibrium was attained. Sometimes the speed was then increased so that the centrifugal field was approximately doubled and a further set of equilibrium photographs was taken. The apparent molecular weight, M, at the centre of the (0·8mm.) column in each channel was calculated from the relation

$$M = \frac{RT}{\omega^2(1-\bar{v}\rho)} \cdot \frac{1}{\bar{r}c_{\bar{r}}} \left(\frac{dc}{dr}\right)_{\bar{r}}$$

where $c_{\bar{r}}$ is the concentration at $\bar{r}$, $(dc/dr)_{\bar{r}}$ the concentration gradient at $\bar{r}$, $\bar{r}$ the distance of the centre of the column from the axis of rotation, ω the angular velocity, ρ the density, and the other symbols have their usual significance. Although $c_{\bar{r}}$ could generally be taken as equal to the initial concentration, c_0, small corrections were incorporated to take account of the differences.

The final equilibrium patterns recorded with interference optics gave the distributions of concentration directly. When the regression of $\ln c$ on r^2 was linear, the statistical regression coefficient was used to calculate M from the expression

$$M = \frac{2RT}{\omega^2(1-\bar{v}\rho)} \cdot \frac{d(\ln c)}{d(r^2)}$$

Gel filtration on a Sephadex G-75 column at various pH *values.* The ratio of elution volume (V_e) of Fab fragment on a Sephadex G-75 column to the void volume (V_0) was measured. The column (3cm. × 137cm.) used throughout the experiments was equilibrated successively with 50mM-NaCl–10mM-tris buffer, pH 7·8, 50mM-NaCl–10mM-sodium acetate buffer, pH 4·5, and 50mM-NaCl–5mM-HCl, pH 2·35, and samples of Fab fragment dialysed against each buffer were passed through the column in the corresponding solvent. A constant V_e/V_0 value of 1·55 was obtained for the Fab fragment regardless of pH, whereas Fc fragments gave values varying as a function of pH, in accord with their behaviour on thin-layer gel filtration described below.

Thin-layer gel filtration on Sephadex G-200. A thin-layer gel of Sephadex G-200 (superfine grade) was prepared on a glass plate (12cm. × 16·5cm.), by the method of Williamson & Allison (1967). A 3% (w/v) suspension of the gel in 0·1M-NaCl was equilibrated at room temperature for at least 3 days. Coated plates were placed in an airtight chamber saturated with moisture, and washed overnight with 25ml. of one of the buffers described above. Solutions of intact IgG and of fragments sFc, stFc and Fab were prepared at concentrations of 0·7–1% in 0·1M-NaCl, but fragments lFc and mFc, because of their low solubilities near neutrality, were dissolved in 0·1M-$NaHCO_3$ at concentrations of 0·4–0·5%. Samples (approx. 1μl.) of these six solutions were placed on each thin-layer plate, giving circles not exceeding 0·2cm. in diameter at 1·3cm. intervals and at least 2·5cm. from the edges of the plate. After a running time of 7–10hr. with a plate angle of 6° at room temperature, the plates were dried in an oven at 60° for 20min. and stained with Amido Black 10B.

The average migration distance ($\bar{R}$) from the origin to the centre of mass-distribution of each stained spot, which was judged by inspection through a pale-blue filter, was measured to the nearest millimetre, and the ratio of $\bar{R}$ of a fragment to $\bar{R}$ of fragment Fab on the same plate was

calculated. The run at each pH was repeated at least five times and an average $\bar{R}/\bar{R}_{Fab}$ value for each sample was obtained. The S.D. for each sample at any particular pH was less than 10%.

RESULTS

With a few exceptions referred to below, the schlieren peaks in velocity experiments were fairly sharp and symmetrical. The values of $S^0_{20,w}$ (Fig. 1) showed a large fall below pH 4 for all the Fc fragments studied, and a moderate fall for intact IgG. There was no appreciable change in $S^0_{20,w}$ of Fab fragment or of tryptic $F(ab)_2$ fragment in the pH range tested.

The results of the most relevant short-column molecular-weight determinations are given in Table 1, and some of the results obtained by interference methods are shown in Fig. 2. The findings may be summarized as follows. Fab fragment gave a molecular weight (Table 1) at low pH and low speed that corresponded to the previous value obtained near neutrality (Charlwood, 1959; cf. Marler, Nelson & Tanford, 1964), but when a higher speed was used the apparent molecular weight fell, indicating some heterogeneity. This was supported by the results shown in Fig. 2(*D*). The value at the higher speed was much nearer the value of 42800 reported by Pain (1963) for rabbit Fab fragment at pH 3·5.

The molecular weight of lFc fragment was unaffected when the pH was lowered from 4·5 to 2·36 (Table 1), despite the substantial fall in $S^0_{20,w}$ under these conditions (Fig. 1*a*). The sedimentation coefficient (2·9s) of lFc fragment at pH 2·4 in the present study is considerably higher than that obtained by Inman & Nisonoff (1966*a*), probably because of the greater charge effect at the lower concentration of salt they used (50mM) and because they did not extrapolate to zero concentration. Similar comments apply to the sedimentation coefficient of IgG at low pH. Although the schlieren curve of lFc fragment in velocity experiments at pH 4·5 was symmetrical, at pH 3·0 there was some indication of a faster-sedimenting shoulder (Fig. 3*a*), and at pH 2·36 there was detectable trailing (Fig. 3*b*).

The large fall in $S^0_{20,w}$ of mFc fragment when the pH was lowered (Fig. 1*a*) was accompanied by a corresponding change in apparent molecular weight (Table 1). At pH 3·6, comparison of apparent molecular weights at two speeds indicated some type of heterogeneity. At pH 2·92, where the value of M was not much more than half its value at pH 5, the more sensitive equilibrium plot (Fig. 2*B*) still showed some curvature.

For fragment sFc there was a fall in $S^0_{20,w}$ between pH 4 and pH 3, but a small increase at even lower pH (Fig. 1*b*). The reason for this rise is not clear, but possibly there is some reassociation. At neutrality the molecular weight was about 44000 (Table 1), but there was some evidence of heterogeneity (see Fig. 2*C*). The molecular weight fell to about 32000 at pH 2·4, where the equilibrium plot was almost linear (Fig. 2*A*). Figs. 3(*c*) and 3(*d*) show ultracentrifuge patterns of sFc fragment.

The variation of $S^0_{20,w}$ with pH for fragment stFc was not large (Fig. 1*b*), nor was there apparently any change in molecular weight (Table 1). Even when 1M-acetic acid was included in the solvent at low pH there was no evidence of dissociation.

Gel filtration of IgG and fragments. The different effects of low pH on the various fragments of IgG were first noticed from the elution positions on a Sephadex G-75 column. At neutral pH, fragments Fab, lFc and mFc were eluted together ahead of fragment stFc, whereas at low pH fragment lFc was eluted first, followed by fragments Fab, mFc (as monomer) and stFc practically together. Of all

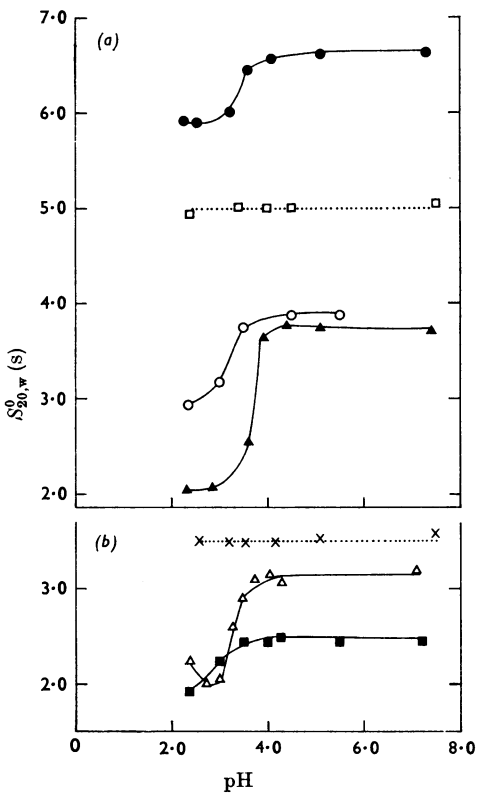

Fig. 1. Sedimentation coefficients of rabbit IgG and fragments derived from it. The solvents and methods were as described in the Materials and Methods section. (*a*) ●, IgG; □, $F(ab)_2$ fragment; ○, lFc fragment; ▲, mFc fragment. (*b*) ×, Fab fragment; △, sFc fragment; ■, stFc fragment.

Table 1. *Molecular weights measured by the short-column method*

For definitions of the fragments, and experimental details, see the text. *M*, Molecular weight.

Fragment	Approx. concn. (mg./ml.)	pH	Speed (rev./min.)	*M*	Speed (rev./min.)	*M*
Fab	1·5	2·30	12590	54100	17980	43700
	3·0	2·30	12590	51400	17980	46400
lFc	1·5	4·50	13410	47300		
	3·0	4·50	13410	49700		
	1·5	2·36	13410	49700		
	3·0	2·36	13410	48700		
mFc	1·5	5·00	12590	44000		
	3·0	5·00	12590	48300		
	1·5	3·60	12590	34900	17980	30400
	3·0	3·60	12590	36500	17980	31700
	2·3	2·92	12590	26300	17980	25600
	4·5	2·92	12590	27800	17980	26600
sFc	1·5	7·05	13410	44700	17980	41800
	3·0	7·05	13410	44300	17980	41800
	1·5	2·40	13410	31400	17980	31800
	3·0	2·40	13410	33100	17980	32400
stFc	1·5	7·20	17980	25800		
	2·5	7·20	17980	25500		
	3·5	7·20	17980	27600		
	1·5	2·40	19160	24100		
	3·0	2·40	19160	22700		

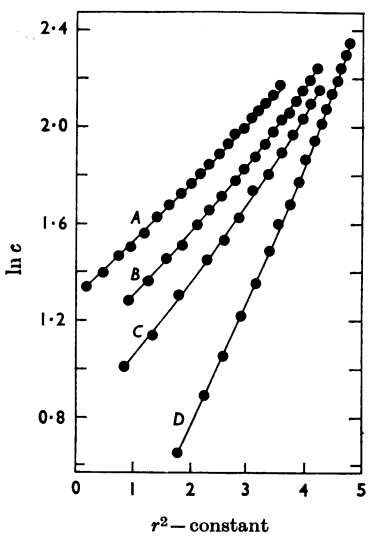

Fig. 2. Sedimentation-equilibrium results for sFc fragment at pH 2·40 (*A*), mFc fragment at pH 2·92 (*B*), sFc fragment at pH 7·05 (*C*) and Fab fragment at pH 2·30 (*D*). Initial concentrations were all approximately 1·6 mg./ml. Solvent details are given in the experimental section. The solid line through set (*A*) is that calculated for a mixture of components (see the text); the other solid lines (*B*, *C* and *D*) are experimental.

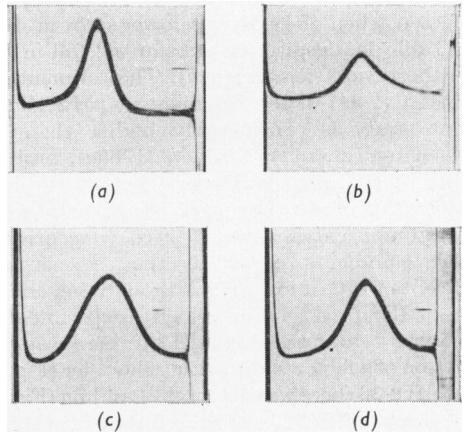

Fig. 3. Ultracentrifuge patterns given by: (*a*) fragment lFc after 110 min. at pH 3·00; (*b*) fragment lFc after 136 min. at pH 2·36; (*c*) fragment sFc after 169 min. at pH 2·79; (*d*) fragment sFc after 169 min. at pH 3·30. The times given refer to the periods at 59780 rev./min. The temperature was approximately 20° in all cases. Solvents are listed in the Materials and Methods section. Sedimentation was from right to left.

these substances, fragment Fab alone showed a constant V_e/V_0 ratio on the Sephadex G-75 column over the range of pH covered. This is consistent

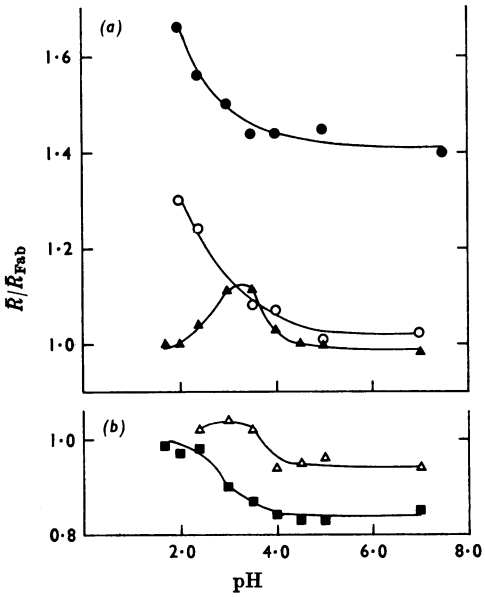

Fig. 4. Mobility on gel filtration of IgG and Fc fragments, expressed relative to that of Fab fragment. The measurements were done in thin layers of Sephadex G-200 in the same solvents as the sedimentation experiments summarized in Fig. 1. For full details, see the text. (*a*) ●, IgG; ○, lFc fragment; ▲, mFc fragment. (*b*) △, sFc fragment; ■, stFc fragment.

with the ultracentrifugal observations, its S value remaining constant and its M value being the same at low and high pH. The distances of migration of other test samples on thin-layer gel filtration were therefore expressed relative to Fab by taking ratios $\bar{R}/\bar{R}_{Fab}$. On thin layers of superfine-grade Sephadex G-200, samples gave fairly compact circular spots at each pH, except that lFc fragment, when applied at concentrations higher than 0·5%, showed elongated spots spreading towards the faster side, indicating the presence of aggregates. The results are summarized in Fig. 4.

DISCUSSION

Several general points about the sedimentation measurements should be made. Obtaining $S^0_{20,w}$ from only three (or even two) points appears justified because in no case was the concentration dependence of S very large, and all the results fitted quite well on smooth curves of S versus pH (Fig. 1). No attempt was made to estimate the effects of charge or non-ideality on apparent molecular weights because there is no general, straightforward method of doing so from the present results and a reasonable interpretation is possible without invoking factors such as these.

In the present study the physical measurements at the highest pH gave values of about 48000 and 46000 for the molecular weights of fragments lFc and mFc respectively, in excellent agreement with figures based on the chemical composition (Utsumi, 1969). The curvature of the equilibrium plot for fragment mFc at pH2·92 (Fig. 2*B*) presumably arises because dissociation is not quite complete. One problem with fragment lFc is its limited solubility at higher pH, but the short-column measurements at pH4·5 at two different concentrations gave no evidence for deviations attributable to this, nor did the solubility limit seem to be reached at the base of the column.

Although the average molecular weight of fragment sFc at neutrality corresponded to what would be expected from the chemical constitution, it was not physically homogeneous at pH2·79 or 3·30 (Fig. 3*c* and 3*d*). However, at pH2·4 it gave an equilibrium plot that seemed practically linear (Fig. 2*A*) and corresponded to an average molecular weight of 31700. On chemical grounds only units of molecular weight about 21000 and 42000 would be expected to exist, and it might be supposed that no mixture of such species, whether in rapid chemical equilibrium with each other or not, could give the points shown in Fig. 2*A*. However, Fujita & Williams (1966) emphasized the insensitivity of conventional equilibrium plots to heterogeneity, a point borne out by the curve shown in Fig. 2*A*. This, although calculated for a mixture of components of molecular weights 21000 and 42000 and fitted to two of the experimental points, passes close to them all. Other assumptions would obviously provide an even better fit, and more detailed studies based on methods such as those of Adams & Williams (1964) or Adams (1965) might clarify the situation. If there were a mixture of monomers and dimers, analysis of the velocity pattern would be expected to show whether there was rapid chemical equilibrium. However, with molecules as small as this it would probably be necessary to make extra assumptions before attempting to decide whether the boundary spreading (Fig. 3*c* and 3*d*) was accounted for by independent or chemically reacting species.

The curves of S versus pH for lFc and mFc fragments (Fig. 1*a*) show a marked difference not only in the lowest S values but also in the maximum slope. The two fragments differ by only ten amino acid residues/chain and the interchain disulphide bridge in the N-terminal portion, so that the difference in sedimentation behaviour must be attributed mainly to the presence or absence of the covalent linkage. The sharp fall in S value for fragment mFc between pH4·5 and 2·4 reflects dissociation, but how conformation change contributes to it is not clear because there is no unambiguous way of interpreting frictional ratios. The

change in S of lFc fragment with pH, on the other hand, is ascribed entirely to a change in conformation.

The molecular weight of stFc fragment at pH 2·4 agrees well with that calculated from chemical data on the basis of the dimeric structure. Since there is no interchain disulphide bridge in this fragment, the resistance to dissociation is attributed to strong non-covalent forces between the chain fragments. Nevertheless, the fall in S, which is regarded as the result of conformational change, is relatively large and the percentage fall is almost the same as that shown by lFc fragment. If the conformational change arises mainly from partial disruption of the quaternary structure, the residual strong non-covalent bonds must be in a relatively small region of the molecule. That these bonds are of a hydrophobic nature is implied by the fact that stFc fragment can be dissociated only by strong denaturing agents such as 40mM-sodium decyl sulphate (Utsumi & Karush, 1965). Of the seven tyrosine residues/chain of the Fc moiety, five are located in the stFc region (Prahl, 1967; Utsumi, 1969), three of them between residues 143 and 177 (counting from the N-terminus) of the amino acid sequence given by Hill, Lebovitz, Fellows & Delaney (1967).

If possible minor changes in partial specific volume with change of pH are neglected and the solvents used are taken as having the same density and viscosity, the Stokes radius (a) is directly proportional to M/S, because $a = M(1-\bar{v}\rho)/6\pi\eta\ NS$, where N is Avogadro's number and η is the solvent viscosity. Thus the values of a for undissociated fragment mFc at neutral pH and for the monomeric form at pH 2·4 differ by only 5%, whereas for fragments lFc and stFc the conformational change when the pH is lowered causes a to increase by 30%.

The constancy of $S^0_{20,w}$ for univalent Fab fragment over the range of pH covered, which is in marked contrast with the behaviour of the Fc moiety, indicates that the association between light chain and Fd fragment (the N-terminal portion of heavy chain) is conformationally stable. Further, pH has little or no effect on the conformation of bivalent F(ab)$_2$ fragment judged by the practically constant S value of F(ab)$_2$ fragment produced by tryptic digestion.

The conformational change of mFc fragment preceding its dissociation is clearly demonstrated by gel filtration. The $\bar{R}/\bar{R}_{Fab}$ versus pH curve for this fragment rose rapidly below pH 4·5 to reach a maximum near pH 3·5. Not only was no aggregation of the fragment indicated in the ultracentrifuge, but equilibrium analysis revealed partial dissociation of the molecules at pH 3·6 (Table 1). The rise of $\bar{R}$ of mFc fragment in this region of pH, like that for IgG or lFc fragment, is therefore attributable exclusively to the change in shape. The process of chain dissociation of mFc fragment is indicated by the fall of the curve between pH 3·5 and 2. Although the value of $\bar{R}/\bar{R}_{Fab}$ reached at pH 2·4 was practically identical with that at neutral pH, there is no contradiction because there is little or no difference between the Stokes radii of fragment mFc dimer at neutral pH and monomer at pH 2·4. These observations confirm that it is the Stokes radius rather than the molecular weight of a protein molecule that determines its gel-filtration behaviour. The desirability of supplementing gel-filtration data with other physical parameters such as S for an unequivocal determination of M must be emphasized (Ackers, 1964; Laurent & Killander, 1964; Siegel & Monty, 1966).

A maximum value of $\bar{R}$ also occurred with sFc fragment but at a slightly lower pH than for mFc fragment, and the subsequent fall of the curve was less conspicuous. The behaviour of sFc fragment, nevertheless, indicates the tendency of the molecule to dissociate at low pH, as found by sedimentation equilibrium. The curve of $\bar{R}/\bar{R}_{Fab}$ for stFc fragment did not quite reach a maximum. The stFc portion is the C-terminal constituent of mFc fragment, which itself easily dissociates. Comparison of the behaviour of fragments mFc, sFc, and stFc on gel filtration and sedimentation clearly demonstrates the decreasing dissociability as the molecule loses its N-terminal portion, i.e. there is a net decrease in repulsive forces resulting from the loss of the N-terminal portions of the chains.

A possible mode of assembly of the Fc moiety in which its halves are symmetrically situated was

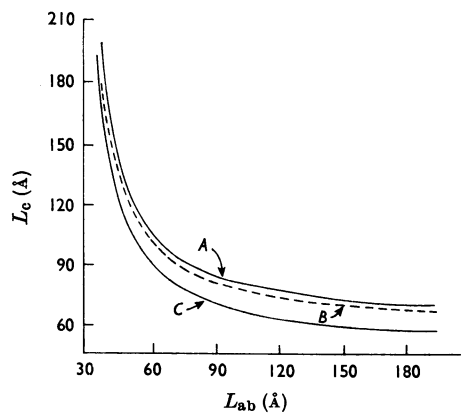

Fig. 5. Possible separations of the Fab units of rabbit IgG from each other (L_{ab}) and from Fc fragment (L_c), as calculated from Eqn. (2) (see the text). Curve A refers to pH 2·4 and curve C to pH 5·0. In both cases $\bar{v}$ was taken as 0·738 and M as 50000 for each unit. The broken curve B was obtained by assuming $\bar{v}$ was increased by 1% at pH 2·4. For definitions and details of calculations, see the text.

suggested in the original model of Porter (1962). The present observations are consistent with this. Only such a symmetrical arrangement would allow the various Fc fragments to be present in stable dimeric forms. The effect of pH on the quaternary structure, resulting in susceptibility of Fc fragment to enzymic attack, would explain the progressive degradation of IgG by which the various types of fragments are obtained.

Phelps & Cann (1957) attributed the behaviour of bovine γ-pseudoglobulin as the pH was lowered to decreased asymmetry, but increased effective volume, and Thorpe & Deutsch (1966) concluded that the behaviour of human IgG at low pH is governed by changes in the Fc portion. To judge whether the behaviour of rabbit IgG in solution is consistent with expectations for a simple symmetrical assembly of one lFc and two Fab hydrodynamic units, calculations were done by using eqn. (15) of Bloomfield (1966), which is a generalization of one derived by Kirkwood (1954). Although more recent work (Bloomfield, Dalton & Van Holde, 1967) implies that the generalized equation is not accurate if the units involved differ greatly in size, this disadvantage would not apply to the present example. Nevertheless, certain discrepancies in fundamental theory that are still unexplained (Bloomfield *et al.* 1967; V. Bloomfield, personal communication to the late Dr R. C. Valentine) must be borne in mind.

If ζ and ζ_1 are the frictional coefficients of the assembly and of unit 1 respectively, the equation relating them can be expressed as

$$\zeta^{-1} = \left(\sum_{l=1}^{n} \zeta_l\right)^{-1}\left\{1 + \left[6\pi\eta\left(\sum_{l=1}^{n} \zeta_l\right)\right]^{-1} \sum_{\substack{l=1 \\ s\neq 1}}^{n} \sum_{s=1}^{n} \zeta_l \zeta_s \langle L_{ls}^{-1}\rangle_{av.}\right\} \quad (1)$$

in which L_{ls} is the distance between the centres of units l and s and the angular brackets denote a time average. For the model under consideration eqn. (1) simplifies to

$$\zeta^{-1} = (2\zeta_{ab} + \zeta_c)^{-1}\{1 + [3\pi\eta(2\zeta_{ab} + \zeta_c)]^{-1} \times [\zeta_{ab}^2/L_{ab} + 2\zeta_{ab}\,\zeta_c/L_c]\} \quad (2)$$

in which ζ_{ab} and ζ_c refer to fragments Fab and lFc respectively, L_{ab} is the separation of the two Fab units, and L_c the separation of the lFc unit from each Fab unit. The frictional coefficients are defined by $\zeta = 6\pi\eta a = M(1-\bar{v}\rho)/NS$. It is assumed that the hydrodynamic drag arising from the links between units is negligible.

If the values of ζ applying under a particular set of conditions are inserted in eqn. (2), a hyperbolic relation between L_{ab} and L_c is obtained (Fig. 5). To determine both L_{ab} and L_c, further information is necessary. The first possibility depends on the assumption that L_{ab} and L_c are the same in two different situations, when their values are defined by the intersection of the appropriate curves. Thus, in Fig. 5, if $\bar{v}$ is 0·738 at both pH values, the extensions of the curves A and C shown intersect when $L_{ab} = 32{\cdot}4$ Å and $L_c = 290$ Å. On the other hand, if v is 1% larger at the lower pH (see Charlwood, 1957), the intersection of the broken curve B and the curve C in Fig. 5 gives $L_{ab} = 37$ Å and $L_c = 185$ Å. The effect of some other variations in the parameters used for calculating L_{ab} and L_c is shown in Table 2. In all cases the relations $M_G = 2M_{ab} + M_c$ and $M_G\bar{v}_G = 2M_{ab}\bar{v}_{ab} + M_c\bar{v}_c$ are satisfied, the subscript G referring to IgG. Because calculations based on the amino acid composition of lFc fragment (Utsumi, 1969) gave $\bar{v} = 0{\cdot}734$, columns based on this are included. The dimensions in Table 2 are

Table 2. *Spatial separations of Fab and lFc units in rabbit IgG, if independent of* pH

All separations (L) are in Ångstrom units. In every case $\bar{v}_G$ at pH 5·0 is 0·738. For IgG S is 6·6 s at pH 5·0 and 5·9 s at pH 2·4; for Fab fragment S is 3·5 s at both pH 2·4 and pH 5·0; for lFc fragment S is 3·8 s at pH 5·0 and 2·9 s at pH 2·4. For full definitions, and for details of methods and calculations, see the text.

$\bar{v}_c$ at pH 5·0		0·738		0·738		0·738		0·734		0·734	
$\bar{v}_c$ at pH 2·4		0·738		0·738 × 1·01		0·738 × 1·02		0·734		0·734 × 1·01	
$\bar{v}_G$ at pH 2·4		0·738		0·738 × 1·01		0·738 × 1·02		0·738		0·738 × 1·01	
$10^{-3} \times M_{ab}$	$10^{-3} \times M_c$	L_{ab}	L_c	L_{ab}	L_c	L_{ab}	L_c	L_{ab}	L_c	L_{ab}	L_c
45	45	29	261	33	166	39	120	29	242	33	157
45	50	31	198	36	140	43	107	31	187	36	134
45	55	33	165	39	124	48	99	33	157	39	119
50	45	31	449	35	232	40	153	31	398	35	217
50	50	32	290	37	184	43	133	33	268	37	175
50	55	34	225	39	158	47	120	35	212	40	151
55	45	33	1085	36	342	41	198	33	844	36	312
55	50	34	468	38	249	44	166	34	418	39	233
55	55	36	319	40	208	47	147	36	295	41	192

Table 3. *Spatial separations of Fab and lFc units in rabbit IgG*

All separations (L) are in Ångstrom units. The parameters are the same as in Table 2, and for tryptic F(ab)$_2$ fragment S is 5·0s at both pH5·0 and pH2·4.

pH		5.0		2·4		2·4	
$\bar{v}_c$		0·738		0·738		0·738×1·01	
$\bar{v}_G$		0·738		0·738		0·738×1·01	
$10^{-3}\times M_{ab}$	$10^{-3}\times M_c$	L_{ab}	L_c	L_{ab}	L_c	L_{ab}	L_c
45	45	69	69	69	83	67	81
45	50	69	73	69	86	67	83
45	55	69	77	69	88	67	85
50	45	77	72	77	90	75	87
50	50	77	76	77	92	75	90
50	55	77	81	77	95	75	92
55	45	84	74	84	96	82	93
55	50	84	79	84	99	82	96
55	55	84	84	84	102	82	99

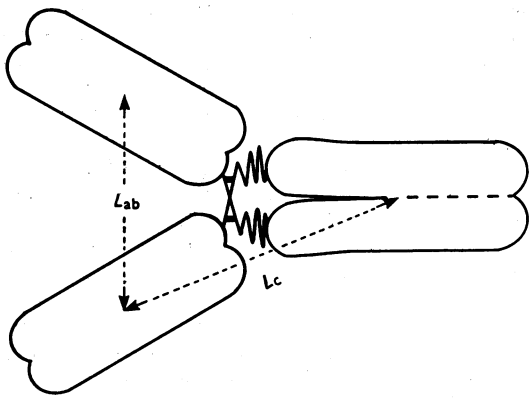

Fig. 6. Schematic structure for rabbit IgG in solution, showing two Fab units L_{ab} Å apart and L_c Å from the Fc unit. The thick lines denote disulphide bridges, and zigzags the extensible portions of the heavy chains. The broken line in the Fc unit indicates the region of strong non-covalent interaction. At neutrality, L_{ab} and L_c are both 76±8Å, but at low pH L_c is increased by 16±4Å. For definitions and details of calculations, see the text.

obviously too critically dependent on some of the variables to be reliable. Moreover, the large values obtained for L_c cast doubt on the main premise that both L_{ab} and L_c are unaffected by fall in pH.

The second alternative requires an independent assessment of L_{ab} or L_c. Provided that the Fab units in whole IgG retain the same relative disposition as in F(ab)$_2$ fragment, the value of L_{ab} can be found from the behaviour of Fab and F(ab)$_2$ fragments, for which eqn. (1) simplifies to

$$\zeta_{ab_2}^{-1} = (2\zeta_{ab})^{-1} + (12\pi\eta L_{ab})^{-1} \qquad (3)$$

where subscript ab_2 refers to F(ab)$_2$ fragment. Then L_c is easily calculated from equation (2). Table 3 shows the results of this approach. Taking $\bar{v}_c = 0{\cdot}734$, rather than 0·738, has virtually no effect on the results, so that columns based on this are omitted. If $S^0_{20,w}$ for tryptic F(ab)$_2$ is taken as 5·1s at neutral pH or 4·9s in acid solution, the results are also not greatly affected. The conclusion from Table 3 is that in neutral solution L_{ab} and L_c are both about 76±8Å, but that at low pH L_{ab} is altered little or not at all, whereas L_c is increased by 16±4Å. From the electron micrographs published by Valentine & Green (1967), estimates of L_{ab} and L_c can be made (approx. 50Å and 60Å respectively).

Variations in L_c can occur if IgG may be represented by the structure shown in Fig. 6, which is similar in general outline to ones proposed by Noelken *et al.* (1965) on the basis of solution properties, and by Valentine & Green (1967) from electron micrographs. It differs from the structure of Noelken *et al.* (1965) in restricting practically all the extensible part of the molecule to the lFc portion. It also contains an indication of that region of the compact part of lFc fragment whose structure is least affected by change of pH. The hypothetical shapes attributed to the units are not to be regarded as implying that these are the true physical shapes, since a particle of given frictional ratio could have an infinite number of shapes. Resort to the conventional representation as ellipsoids (Oncley, 1941) gave the dimensions shown in Table 4 for fragment Fab or Fc at pH5·0. In no case is it possible to make an assembly of these ellipsoids with major axes co-planar and separations consistent with those of Table 3 for the corresponding conditions. There is a suggestion from electron microscopy that the units may approximate to squat cylinders (Valentine & Green, 1967), but apparently the frictional ratios of such bodies have not been estimated either experimentally or theoretically. The lowest ratio of length to diameter of cylinders so far dealt with is 2:1 (Bloomfield *et al.* 1967).

Table 4. *Dimensions from the conventional expression for prolate ellipsoids*

The calculated lengths of the axes of revolution ($2a$) and equatorial axes ($2b$) of prolate ellipsoids of substances of different molecular weights (M), partial specific volumes ($\bar{v}$) and sedimentation coefficients (S) are shown.

			Length of axes (Å)			
	Hydration ...		0%		30%	
$10^{-3} \times M$	$\bar{v}$	S(s)	a	b	a	b
45	0·738	3·5	69	13·8	50	16·2
50	0·738	3·5	83	13·3	65	15·0
55	0·738	3·5	102	12·6	80	14·2
45	0·738	3·8	56	15·3	34	19·8
50	0·738	3·8	71	14·4	49	17·4
55	0·738	3·8	84	13·8	63	16·0
45	0·734	3·8	59	14·8	40	18·1
50	0·734	3·8	71	14·3	53	16·6
55	0·734	3·8	87	13·6	66	15·6
45	0·738	2·9	106	11·1	90	12·1
50	0·738	2·9	122	10·9	98	12·2
55	0·738	2·9	143	10·6	115	11·8

The general conclusion is that the sedimentation and gel-filtration behaviour of rabbit IgG is consistent with a structure of the type shown in Fig. 6, but the separations of the Fab and Fc units in solution are greater than those deduced from electron micrographs. It is unfortunate that only the dried form of the protein is amenable to electron microscopy.

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