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, 1966a,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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DEAE-cellulose (W. and R. Balston, Maidstone, Kent) with 8 mm-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 IgF fragment above, was further treated with pepsin (twice crystallized; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) for 24 hr at pH 4-5 and 37° in 0-1 M-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 0-1 M-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 10 mM-HCl at a protein concentration of 1%. The enzyme/ 

IgG ratio was 1:50 (w/v). Digestion was for 24 hr at 37°, the pH being maintained between 2-2 and 2-4 by addition of 10 mM-HCl. The pH was then adjusted to 7-5 with 2 M-tris, and the ensuing cloudiness was cleared by repeated freezing (in a solid CO2–acetone bath) and thawing. The digest was placed on a column of Sephadex G-150 (Pharmacia, Uppsala, Sweden) in 20 mM-sodium phosphate–0-2 M NaCl buffer, pH 7-8, containing 2 mM-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 14 M-mercaptoethanol.

Preparation of solvents and protein solutions. To minimize changes of environment, apart from pH, the solvent was usually 0-1 M-NaCl containing a low-capacity buffer. Between pH 3-5 and pH 5-5 this was 10 M-acetic acid, adjusted with NaOH. Below pH 3-5, HCl alone was added to the NaCl, whereas above pH 5-5 the solvent contained 10 mM-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 59780 rev./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 546 mµ. The cells were either 3 mm. or 12 mm., Kel-F centrepieces being used below pH 5. Synthetic-boundary cells were used for samples of low protein concentration or low sedimentation coefficient to improve accuracy. All sedimentation coefficients were corrected to water at 20° in the usual way and expressed in Svedberg units. In the calculations 6 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 ΔS0,2 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, 1967a,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 16 min. 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, Mc, at the centre of the (0-8 mm.) column in each channel was calculated from the relation

\[ M = \frac{RT}{\omega^2(1-\epsilon)} \left( \frac{1}{\eta_c(\frac{dc}{dr})} \right) \]

where \( c \) is the concentration at \( r \), \( (dc/dr) \) the concentration gradient at \( r \), \( \epsilon \) the distance of the centre of the column from the axis of rotation, \( \omega \) the angular velocity, \( \rho \) the density, and the other symbols have their usual significance. Although \( \epsilon \) 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

\[ M = \frac{2RT}{\omega^2(1-\epsilon)} \left( \frac{d(ln c)}{d(r^2)} \right) \]

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 (5 cm. x 137 cm.) used throughout the experiments was equilibrated successively with 50 mM-NaCl–10 mM-tris buffer, pH 7-8, 50 mM-NaCl–10 mM-sodium acetate buffer, pH 4-5, and 50 mM-NaCl–5 mM-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 Fe 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 (12 cm. x 16-5 cm.), by the method of Williamson & Allison (1967). A 3% (w/v) suspension of the gel in 0-1 M-NaCl was equilibrated at room temperature for at least 3 days. Coated plates were placed in an air-tight chamber saturated with moisture, and washed overnight with 25 ml. 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 0-1 M-NaCl, but fragments IgF and mFc, because of their low solubilities near neutrality, were dissolved in 0-1 M-NaHCO3 at concentrations of 0-4–0-5%. Samples (approx. 1 µl.) of these six solutions were placed on each thin-layer plate, giving circles not exceeding 2 cm. in diameter at 1-3 cm. intervals and at least 2-5 cm. from the edges of the plate. After a running time of 7–10 hr. with a plate angle of 6° at room temperature, the plates were dried in an oven at 60° for 20 min. and stained with Amido Black 10B.

The average migration distance \( (R) \) from the origin to the centre of mass-distribution of each stained spot was judged by inspection through a pale-blue filter, was measured to the nearest millimetre, and the ratio of \( R \) to a fragment of \( R \) of fragment Fab on the same plate was
calculated. The run at each pH was repeated at least five times and an average $R_i/R_{iab}$ 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 $S_{20,w}^0$ (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 $S_{20,w}^0$ of Fab fragment or of trypptic 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 IFc fragment was unaffected when the pH was lowered from 4-5 to 2-36 (Table 1), despite the substantial fall in $S_{20,w}^0$ under these conditions (Fig. 1a). The sedimentation coefficient (2-9s) of IFc fragment at pH 2-4 in the present study is considerably higher than that obtained by Inman & Nisonoff (1966a), 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 IFc fragment in velocity experiments at pH 4-5 was symmetrical, at pH 3-0 there was some indication of a faster-sedimenting shoulder (Fig. 3a), and at pH 2-36 there was detectable trailing (Fig. 3b).

The large fall in $S_{20,w}^0$ of mFc fragment when the pH was lowered (Fig. 1a) 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. 2B) still showed some curvature.

For fragment sFc there was a fall in $S_{20,w}^0$ between pH 4 and pH 3, but a small increase at even lower pH (Fig. 1b). 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. 2C). The molecular weight fell to about 32000 at pH 2-4, where the equilibrium plot was almost linear (Fig. 2A). Figs. 3(c) and 3(d) show ultracentrifuge patterns of sFc fragment.

The variation of $S_{20,w}^0$ with pH for fragment stFc was not large (Fig. 1b), 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.

**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; ○, IFc 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.

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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 $V_e/V_o$ 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 Fe 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; ○, lFe fragment; △, mFe fragment. (b) Δ, sFe fragment; ■, stFe fragment.

DISCUSSION

Several general points about the sedimentation measurements should be made. Obtaining $S_{20, w}^{o}$ 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 pH 2-92 (Fig. 2B) 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 pH 4-5 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 pH 2-79 or 3-30 (Fig. 3c and 3d). However, at pH 2-4 it gave an equilibrium plot that seemed practically linear (Fig. 2A) 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. 2A. However, Fujita & Williams (1966) emphasized the insensitivity of conventional equilibrium plots to heterogeneity, a point borne out by the curve shown in Fig. 2A. 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. 3c and 3d) was accounted for by independent or chemically reacting species.

The curves of $S$ versus pH for lFc and mFc fragments (Fig. 1a) 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 pH 4-5 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 3:4 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 40 mM-sodium decyl sulphate (Utsumi & Karush, 1965). Of the seven tyrosine residues/chain of the Fe moiety, five are located in the stFc region (Prahlf, 1967; Utsumi, 1969), three of them between residues 143 and 177 (counting from the N-term 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 ($\alpha$) is directly proportional to $M/S$, because $\alpha = M(1 - \bar{v}_p)/6\pi\gamma NS$, where $N$ is Avogadro's number and $\gamma$ is the solvent viscosity. Thus the values of $\alpha$ 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 $\alpha$ to increase by 30%.

The constancy of $S_{20, w}^\infty$ for univalent Fab fragment over the range of pH covered, which is in marked contrast with the behaviour of the Fe 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 trypic digestion.

The conformational change of mFc fragment preceding its dissociation is clearly demonstrated by gel filtration. The $\overline{R}/\overline{R}_{Fab}$ 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 $\overline{R}$ 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 $\overline{R}/\overline{R}_{Fab}$ 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 $\overline{R}$ 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 $\overline{R}/\overline{R}_{Fab}$ 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 Fe moiety in which its halves are symmetrically situated was
suggested in the original model of Porter (1962). The present observations are consistent with this. Only such a symmetrical arrangement would allow the various Fe fragments to be present in stable dimeric forms. The effect of pH on the quaternary structure, resulting in susceptibility of Fe 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 Fe portion. To judge whether the behaviour of rabbit IgG in solution is consistent with expectations for a simple symmetrical assembly of one IFc 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 $\zeta$ and $\zeta'$ are the frictional coefficients of the assembly and of unit I respectively, the equation relating them can be expressed as

$$\zeta^{-1} = \left( \sum_{i=1}^{n} \zeta_i \right)^{-1} \left[ 1 + 6\pi \eta \left( \sum_{i=1}^{n} \zeta_i \right)^{-1} \sum_{i=1}^{n} \sum_{s=1}^{s} \zeta_i \zeta_s \langle L_{is}^{-1} \rangle_{av} \right]$$

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

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

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

If the values of $\zeta$ 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 $L_{ab} = 32.4$ and $L_{c} = 29.0$. 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 $L_{ab} = 37.3$ and $L_{c} = 185.3$. 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 $M_{G} \equiv 2M_{ab} \equiv M_{ab} + M_{c} \equiv$ are satisfied, the subscript G referring to IgG. Because calculations based on the amino acid composition of IFc fragment (Utsumi, 1969) gave $\bar{v} = 0.734$, columns based on this are included. The dimensions in Table 2 are

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

<table>
<thead>
<tr>
<th>$\bar{v}$ at pH 5.0</th>
<th>$\bar{v}$ at pH 2.4</th>
<th>$\bar{v}$ at pH 5.0</th>
<th>$\bar{v}$ at pH 2.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.738</td>
<td>0.738</td>
<td>0.738</td>
<td>0.734</td>
</tr>
<tr>
<td>0.738 × 10^{-3} M_{ab}</td>
<td>0.738 × 10^{-3} M_{c}</td>
<td>0.738 × 10^{-3} M_{ab}</td>
<td>0.738 × 10^{-3} M_{c}</td>
</tr>
<tr>
<td>45</td>
<td>45</td>
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</tr>
<tr>
<td>50</td>
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</tr>
</tbody>
</table>

For all separations ($L$) in Ångström units. In every case $\bar{v}$ 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 IFc 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.
Table 3. Spatial separations of Fab and Fc units in rabbit IgG

<table>
<thead>
<tr>
<th>pH</th>
<th>5.0</th>
<th>2.4</th>
<th>2.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \bar{v}_G )</td>
<td>0.738</td>
<td>0.738</td>
<td>0.738 x 1.01</td>
</tr>
<tr>
<td>( \bar{v}_c )</td>
<td>0.738</td>
<td>0.738</td>
<td>0.738 x 1.01</td>
</tr>
<tr>
<td>( 10^{-3} \times M_{ab} )</td>
<td>( 10^{-3} \times M_c )</td>
<td>( L_{ab} )</td>
<td>( L_c )</td>
</tr>
<tr>
<td>45</td>
<td>45</td>
<td>69</td>
<td>69</td>
</tr>
<tr>
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<td>84</td>
<td>84</td>
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</tbody>
</table>

All separations (\( L \)) are in Ångstrom units. The parameters are the same as in Table 2, and for tryptic \( \text{F(ab)}_2 \) fragment \( S \) is 5.08 at both pH 5.0 and pH 2.4.

Fig. 6. Schematic structure for rabbit IgG in solution, showing two Fab units \( L_{ab} \) apart and \( L_c \) 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 noncovalent interaction. At neutrality, \( L_{ab} \) and \( L_c \) are both 76±8 Å, but at low pH \( L_c \) is increased by 16±4 Å. For definitions and details of calculations, see the text.

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 \( \text{F(ab)}_2 \) fragment, the value of \( L_{ab} \) can be found from the behaviour of Fab and \( \text{F(ab)}_2 \) fragments, for which eqn. (1) simplifies to

\[
L_{ab}^{-1} = \left(2L_{ab}^{-1}\right) + \left(12\pi\eta L_{ab}^{-1}\right)
\]

where subscript \( ab \) refers to \( \text{F(ab)}_2 \) fragment. Then \( L_c \) is easily calculated from equation (2).

Table 3 shows the results of this approach. Taking \( \bar{v}_c = 0.734 \), rather than 0.738, has virtually no effect on the results, so that columns based on this are omitted. If \( S_{90w} \) for tryptic \( \text{F(ab)}_2 \) is taken as 5-18 at neutral pH or 4-9 s 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±8 Å, but that at low pH \( L_{ab} \) is altered little or not at all, whereas \( L_c \) is increased by 16±4 Å. From the electron micrographs published by Valentine & Green (1967), estimates of \( L_{ab} \) and \( L_c \) can be made (approx. 50 Å and 60 Å 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 Fc portion. It also contains an indication of that region of the compact part of Fc 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 pH 5.0. 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).
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 Fe 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.

We are indebted to Mrs D. Dwyer and Mr S. Gresswell for excellent technical assistance and to the late Dr R. C. Valentine for helpful discussions. S.U. is grateful for the award of a Wellcome Research Fellowship that enabled him to participate in this work.

REFERENCES


