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Stepwise Cleavage of Rabbit Immunoglobulin G by Papain and Isolation of Four Types of Biologically Active Fc Fragments

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Four types of Fc fragments of different sizes were isolated by papain treatment of rabbit immunoglobulin G under various conditions and by subsequent chromatographic procedures. 1. Brief digestion at neutral pH without reduction produced a molecule in which the Fab and Fc fragments were still linked by a pair of labile disulphide bridges, and the Fc fragment released by cleaving these bonds, called lFc fragment, contained a portion of the 'hinge' region including an interchain disulphide bridge. Both complement-binding and guinea-pig skin-binding activities were retained by this fragment, which had mol. wt. 48000. 2. Prolonged digestion at neutral pH of immunoglobulin G whose labile inter-heavy-chain disulphide bridges had been reduced removed the 'hinge' region, giving mFc fragments (mol. wt. 46000), which lacked the capacity to bind guinea-pig skin but retained the antigenic as well as the complement-binding activities of lFc fragment completely. 3. Digestion at pH 5·0 yielded a smaller fragment, sFc (mol. wt. 40000), which was no longer able to bind complement. Though the antigenic structure was intact, sFc fragment was curiously unable to precipitate with antibodies to the *N*-terminal determinants. 4. Fragment stFc (mol. wt. 25000), representing the *C*-terminal portion of Fc fragment, was formed from all the larger fragments by digestion at pH 4·5. Only the *C*-terminal antigenic determinants were retained by stFc fragment.

Since the original work of Porter (1959), the papain fragments of IgG† Fab and Fc, have been most valuable tools in a variety of studies. (The nomenclature of these fragments is that recommended by the World Health Organisation, 1964.) They are nevertheless not entirely well-defined, as expected from the nature of the enzyme, and are still too large for detailed investigation of the structural basis of biological functions. More specific methods for the cleavage have as yet given no additional information about the function of antibodies. Despite its broad specificity papain was again used in the present study, since it produces biologically active fragments.

The present study was directed to the preparation and isolation of a series of fragments of Fc fragment from rabbit IgG by controlling the digestion conditions and developing chromatographic procedures. By their physical and gross chemical properties these products were classified into four distinct types. By using these fragments, an investigation was made of the location of sites responsible for some of the biological effects that had been shown to be associated with Fc fragment, such as antigenic activity (Porter, 1959), skin sensitization in the guinea pig (Ovary & Karush, 1961) and complement-binding (Taranta & Franklin, 1961; Ishizaka, Ishizaka & Sugahara, 1962).

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†Abbreviations: IgG, immunoglobulin G; SCMC, *S*-carboxymethylcysteine; $C'H_{50}$, 50% haemolytic titre of complement according to the definition by Mayer (1961).

MATERIALS AND METHODS

General methods

Preparation of rabbit IgG. Crude rabbit IgG, obtained from pooled rabbit (New Zealand White) serum by precipitation with 40% satd. $(NH_4)_2SO_4$, was passed through a column of Whatman DEAE-cellulose equilibrated with 20mM-sodium phosphate buffer, pH 7·2.

Reduction and alkylation of disulphide bridges. For limited reduction of the labile disulphide bridges (mainly the inter-heavy-chain disulphide bridges) of rabbit IgG, IgG (2%, w/v) was incubated with 14mM-2-mercaptoethanol, pH 8·0, in an atmosphere of N_2 for 2hr. Alkylation was done subsequently with 16mM-iodoacetic acid (twice recrystallized from toluene) in 0·2M-tris–HCl buffer, pH 8·2, for 5hr. For alkylation with radioactive iodoacetic acid,

a solution of iodo[2-^{14}C]acetic acid (0·14 mc/m-mole) was prepared by dilution of iodo[2-^{14}C]acetic acid supplied by The Radiochemical Centre (Amersham, Bucks.) (7 mc/m-mole) with carrier. The reduced alkylated samples were extensively dialysed against 5 mM-tris–HCl buffer, pH 8, and, for radioactivity measurements, finally against 10 mM-acetic acid. The SCMC content was determined from the radioactivity measured in a Packard Tri-Carb scintillation spectrometer, as described by Utsumi & Karush (1967).

Extensive reduction of the disulphides was carried out with 0·56 M-2-mercaptoethanol in the presence of 7 M-guanidine hydrochloride for 5 hr. at pH 8·0.

Determination of thiol groups. Protein thiol groups were titrated with 5,5′-dithiobis(2-nitrobenzoic acid) (kindly provided by Dr D. Givol of the Weizmann Institute of Science, Rehovoth, Israel) by the method of Ellman (1959). Portions of samples, containing 2–25 mg. of protein, were removed from the reduction mixtures, cooled and precipitated with 50 vol. of ice-cold aq. 5% (w/v) trichloroacetic acid (Miller & Metzger, 1965). The precipitate, after being washed four times with the same volume of 5% (w/v) trichloroacetic acid, was quickly dissolved in 5–10 ml. of 0·1 M-sodium decyl sulphate in 0·3 M-tris–HCl buffer, pH 8·0, and portions (2·5 ml.) were immediately mixed with 0·05 ml. of 10 mM-5,5′-dithiobis(2-nitrobenzoic acid). The pure preparation of sodium decyl sulphate was generously provided by Dr F. Karush of the University of Pennsylvania. Portions without 5,5′-dithiobis(2-nitrobenzoic acid) served for the determination of protein by E_{280}. Unreduced proteins were also treated with trichloroacetic acid and assayed for reactive free thiol groups under the same conditions.

Dissociation of Fc fragments at pH 2·35. The presence or absence of interchain disulphide bridges in Fc fragments (lFc and mFc fragments) was tested by the dissociation into monomers at pH 2·35 by the method of Inman & Nisonoff (1966). A column (1·6 cm. × 140 cm.) of Sephadex G-75 (Pharmacia, Uppsala, Sweden) was equilibrated with 50 mM-NaCl adjusted to pH 2·35 with HCl and the elution volumes were measured for lFc fragment and for lFc fragment that had been reduced and alkylated. Average elution volumes of 122 ml. and 147 ml. were obtained for the intact (non-acid-dissociated) and the reduced alkylated (acid-dissociated) fractions respectively. The elution volume for mFc fragment, which has a similar molecular weight to that of lFc fragment but lacks the interchain disulphide bridge, was identical with that for reduced alkylated lFc fragment.

Other analytical methods. Protein concentrations were determined from E_{280} in 0·1 M-sodium decyl sulphate, pH 8·0. The extinction coefficient, $E^{1\%}_{1\,cm.}$ at 280 mμ (determined by the micro-Kjeldahl method) was 14·0 for intact IgG, 15·0 for Fab fragment, 11·5 for lFc and mFc fragments, 13·5 for sFc fragment and 16·7 for stFc fragment. Hexose and hexosamine contents were determined as described by Utsumi & Karush (1965). Amino acid analysis was performed with a Spinco amino acid analyser or an EEL amino acid analyser, by the method of Spackman, Stein & Moore (1958). Horizontal starch-gel electrophoresis was carried out in 50 mM-glycine–NaOH buffer, pH 8·9, with 0·3 M-boric acid–50 mM-NaOH in the electrode vessels. The running time was 5–8 hr. at 6·25 v/cm. at room temperature.

DEAE-cellulose chromatography of Fc fragments. A jacketed column (1 cm. × 35 cm.) of Whatman DEAE-cellulose (batch No. DE100F1) was equilibrated with 8 mM-tris–HCl buffer, pH 8·0, for the chromatography of 100–500 mg. of protein. The following elution scheme was used: (1) 400 ml. of 8 mM-tris–HCl buffer, pH 8·0, at 37°; (2) a linear concentration gradient of 8–33 mM-tris–HCl buffer, pH 8·0, developed in a volume of 700 ml., at 45°; (3) at 37°, a concentration gradient of tris–HCl, pH 8·0, delivered by a Varigrad apparatus with 110 ml. of 33 mM-, 33 mM-, 67 mM-, 67 mM-, 67 mM-, 0·17 M-, 0·17 M-, 0·5 M-, and 0·5 M-tris–HCl respectively in compartment nos. (1)–(9). A flow rate of 60 ml./hr. was used throughout, and fractions (8 ml.) were collected.

Preparation of Fc fragments

The following procedures, unless otherwise stated, were used for the preparation of four types of Fc fragments (lFc, mFc, sFc and stFc) in the present study. The preparation and isolation methods were guided by the criteria listed in Table 1, as well as by the characteristic electrophoretic behaviour of these fragments.

Preparation of lFc fragment by limited papain digestion of IgG. Rabbit IgG (1 g.) in 50 mM-sodium phosphate buffer, pH 7·5, containing 2 mM-EDTA was incubated for 10–20 min.

Table 1. *Properties used as criteria for the preparation of Fc fragments*

Fractions overlapping with Fab in gel filtration on Sephadex G-75 or G-150 were conventionally called '3s fraction'. Actual sedimentation coefficients ($S^0_{20,w}$) were: 3·8s for lFc fragment, 3·7s for mFc fragment, 3·15s for sFc fragment, and 2·4s for stFc fragment (Charlwood & Utsumi, 1969). Data for the approx. mol. wt. and dissociability of fragments are taken from Charlwood & Utsumi (1969). The concentrations of tris–HCl buffers, pH 8·0, that eluted the fractions from DEAE-cellulose were: I, 8 mM; II, 8–33 mM; III, 33 mM–0·5 M; see the Materials and Methods section.

Fragment	Fraction in gel filtration	Mol. wt.	Dissociation at pH 2·4	Fraction in DEAE chromatography	Crystallization	Precipitation: With anti-lFc absorbed with stFc	Precipitation: With anti-stFc
lFc	3s	48000	Non-dissoc.	II	Yes	Intact	Intact
mFc	3s	46000	Dissoc.	I	Yes	Intact	Intact
sFc	3s	40000	Partially dissoc.	III	No	Inhibitory	Intact
stFc	2·4s	25000	Non-dissoc.	III	Yes	None	Intact

at 37° with crystalline papain (10mg.; twice crystallized; Koch–Light Laboratories Ltd., Colnbrook, Bucks.) in the absence of reducing agent, and digestion was stopped by cooling in an ice bath. The product was resolved into 7s, 5s, and 3s fractions by gel filtration on Sephadex G-150 (Fig. 1*a*). The freshly prepared 7s fraction with its associated protein aggregate (10s in Fig. 1*a*) was treated with 14mM-2-mercaptoethanol, pH8·0, for 2hr. and, without alkylation, fractionated on Sephadex G-150 (Fig. 1*b*). Most of the protein had been degraded into 3s and a small amount of 5s fragments (cf. Nelson, 1964) with average recoveries of 85% and 12% of the total protein respectively. The 3s fragments were dialysed against 5mM-tris–HCl buffer, pH8·0, at 4° and the crystals formed were collected by centrifugation. The crystals, after being washed with the buffer, were dissolved in 0·1M-sodium acetate buffer, pH4·0, and allowed to recrystallize by repeated dialysis as above. Most of the protein in this preparation failed to dissociate at pH2·35. The Fc fragment thus obtained was designated lFc fragment, the largest fragment.

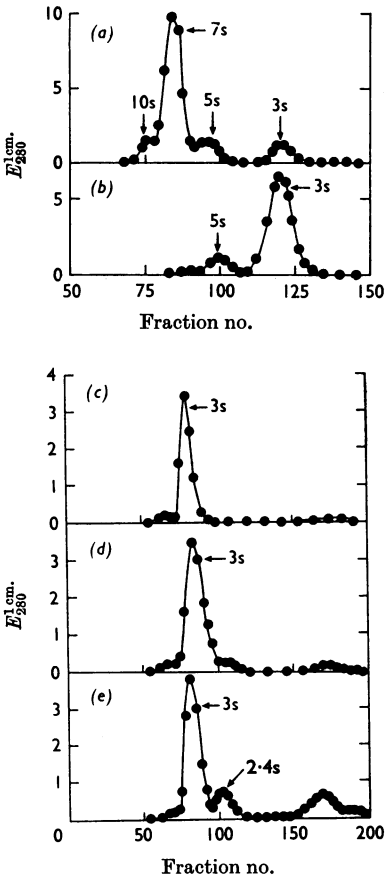

Fig. 1. Gel-filtration diagrams of various papain digests of IgG. (*a*) Limited papain digestion of IgG; 7s fraction = presplit molecule. (*b*) Products of reductive cleavage of the above 7s fraction; 3s fraction = lFc and Fab fragments. (*c*) Products of 24hr. digestion at pH7·5 in the presence of reducing agent; 3s fraction = mFc and Fab fragments. (*d*) Products of digestion of mildly reduced alkylated IgG at pH5·0 in the absence of reducing agent; 3s fraction = sFc and Fab fragments. (*e*) IgG digested at pH4·5 in the presence of reducing agent; 3s fraction = Fab fragment, 2·4s fraction = stFc fragment. In (*a*) and (*b*), fractions (8ml.) were collected from a column (3·2cm. × 176cm.) of Sephadex G-150 in 20mM-sodium phosphate–0·2M-NaCl–2mM-EDTA, pH7·7; in (*c*), (*d*) and (*e*), fractions (6·5ml.) were collected from a column (3·2cm. × 137cm.) of Sephadex G-75 in the same solvent.

Preparation of crude mFc fragment by prolonged digestion at pH7·5. Digestion of IgG was continued for 24hr. in the presence of 14mM-2-mercaptoethanol at 37° in 50mM-sodium phosphate buffer, pH7·5, containing 2mM-EDTA. The Fc fragment was crystallized from the 3s fraction obtained by gel filtration (Fig. 1*c*), and recrystallized in a similar manner to that for lFc fragment except that the crystals were dissolved at pH5·0.

Purification of mFc fragment by DEAE-cellulose chromatography. When a mixture of fragments lFc and mFc (7:3) was chromatographed on DEAE-cellulose by the procedure described above (Fig. 2*a*), the first step with 8mM-tris–HCl buffer eluted acid-dissociable protein alone (fractions Ia and Ib), whereas the second step with 33mM-tris–HCl gave fractions (IIa and IIb) that contained non-acid-dissociable protein. Chromatography of the crude mFc fragment also separated these two types of fragments (Fig. 2*b*). The first two peaks (Ia and Ib), which lacked the interchain disulphide bridge, were rechromatographed, and usually combined as purified mFc fragment. The smaller fragments, sFc and stFc, were not eluted by these two steps of chromatography but only by the third elution step.

Preparation of sFc fragment by papain digestion at pH5·0. A sample of IgG that had been reduced with 14mM-2-mercaptoethanol and alkylated was dialysed against 50mM-sodium acetate buffer, pH5·0, for 24hr. before the digestion. Crystalline papain (10mg.) in 50mM-sodium phosphate buffer, pH7·5, containing 2mM-EDTA, (2ml.) was incubated with 14mM-2-mercaptoethanol for 30min. at room temperature. After removal of the reducing agent by passage through a column (1·3cm. × 50cm.) of Sephadex G-25 (coarse bead grade) in 50mM-sodium acetate buffer, pH5·0, the activated papain was mixed with the reduced alkylated IgG (1g.) and digestion was continued for 20–24 hr. at 37° and pH5·0. The gel-filtration diagram of a 20hr. digest is shown in Fig. 1(*d*). The 3s fraction contained both Fab and Fc fragments. The latter was only partially precipitated with anti-(lFc fragment) absorbed with stFc fragment, but strongly inhibited the precipitin reaction with lFc fragment. This type of Fc fragment was called sFc.

Purification of sFc fragment. The papain digest above was placed on a column (2·2cm. × 35cm. for 1g. of protein) of CM-cellulose in 10mM-sodium acetate buffer, pH5·4. Elution was done first with the initial buffer until the protein concentration of the eluate reached 0·1% or less, and then with 0·4mM-sodium acetate buffer, pH5·4. The second fraction, a mixture of Fab and Fc fragments in which 70% of the total protein was recovered, was dialysed against 16·7mM-tris–HCl buffer, pH8·0, and placed on a column (2·2cm. × 25cm.) of DEAE-cellulose in the same buffer. Elution was carried out with successive 250ml. volumes of 33mM- and 0·3M-tris–HCl buffer, pH8·0. Fragment sFc

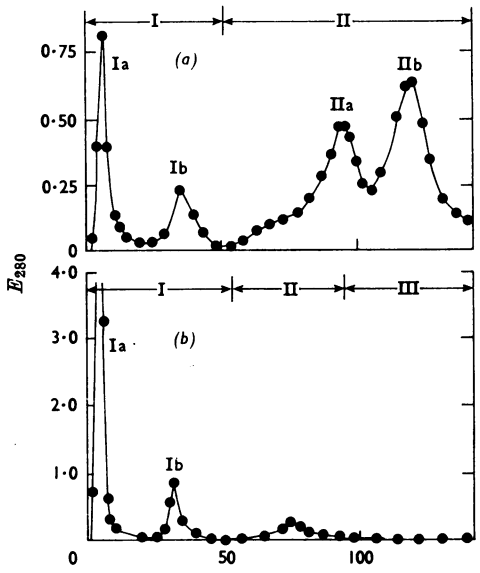

Fig. 2. DEAE-cellulose chromatography of fragments lFc and mFc. (*a*) Chromatography of a mixture of fragments lFc and mFc, obtained from a 24 hr. digestion of IgG with papain in the absence of reducing agent at pH 7·5. A sample (350 mg. of protein) was applied to a column of (1 cm. × 35 cm.) DEAE-cellulose. Elution was done as follows: I, 8 mM-tris–HCl buffer, pH 8·0, at 37°; II, linear concn. gradient 8–33 mM-tris–HCl, pH 8·0, at 45°. (*b*) Chromatography of crude mFc fragment. The same column was used as above, for 220 mg. of protein. Elution was done as follows: I, 8 mM-tris–HCl, at 37°; II, 33 mM-tris–HCl, at 45°; III, stepwise concn. gradient 33 mM–0·5 M-tris–HCl, pH 8·0, at 37°.

was entirely recovered in the second fraction, which contained approx. 40% of the total protein, but Fab fragment was still present. Finally the crude sFc fragment was passed through two consecutive columns (each (3 cm. × 140 cm.) of Sephadex G-75 in 0·2 M-NaCl–2 mM-EDTA–20 mM-sodium phosphate buffer, pH 7·7. Two peaks emerged; the first contained fragment Fab and fragment mFc, if any, and the second sFc fragment alone.

Preparation of stFc fragment by papain digestion at pH 4·5. Rabbit IgG was incubated with papain (1%, w/w) in 2·8 mM-2-mercaptoethanol–50 mM-sodium acetate buffer, pH 4·5, at 37° for 20–24 hr. After being adjusted to pH 8·0, the digest was fractionated with Sephadex G-75 (Fig. 1*e*). The first peak contained only Fab fragment, but the second (2·4 S) fraction contained a fragment designated stFc fragment, analogous to Pep-III′ fragment (Utsumi & Karush, 1965).

Assays of biological activities

Preparation of antisera. Antisera to intact rabbit IgG and to Fab, lFc, sFc and stFc fragments were prepared by immunizing goats with purified antigens. Generally the initial injection was made with 4–5 mg. of antigen/goat and 30 days later the second injection was made with 2 mg., both with Freund's complete adjuvant and intramuscularly. Antiserum to the *N*-terminal antigenic determinants of Fc fragment, which are absent from the stFc region of the molecule, was prepared by the absorption of anti-(lFc fragment) with stFc fragment at equivalence.

Quantitative precipitin reactions. In a final volume of 0·4 ml., 0·2 ml. of antiserum was mixed with various amounts of antigen and incubated for 3 days at 4°. For the inhibition experiments, increasing amounts of sFc fragment were added to the equivalence mixture of lFc (1·2 nmoles) and anti-(lFc fragment) absorbed with stFc fragment (0·2 ml.), to give a final volume of 0·4 ml. In both cases the resulting precipitates were centrifuged off, washed three times, dissolved in 3 ml. of 0·1 M-sodium decyl sulphate–0·3 M-tris–HCl buffer, pH 8·0, and E_{280} was measured.

Reverse passive cutaneous anaphylaxis test. This was performed by the method of Ovary (1964). For reverse passive cutaneous anaphylaxis, Hartley-strain albino guinea pigs (300–400 g.) were injected intracutaneously with samples to be tested (0·1 ml.). After 4 hr., the animals received 0·6 ml. of undiluted goat anti-(stFc fragment) serum–aq. 1% solution of Evans Blue (1:1, v/v) intravenously. Anti-(stFc fragment) reacted *in vitro* with all of the Fc fragments as well as intact IgG, as described later. The results from animals that gave a negative response to IgG (0·1 μg. in 0·1 ml.) were excluded.

Complement-fixation tests. The capacity of Fc fragments to bind complement was assayed indirectly by the anti-complementary activity. The entire experiment below was carried out in the iso-osmotic veronal-buffered saline described by Mayer (1961).

Samples (50 μg. of protein) were mixed with various amounts (0 or 10–150 μg.) of uniform polystyrene latex particles of 88 nm. diameter (Dow Chemical Co., Midland, Mich., U.S.A.; obtained through the courtesy of Dr J. H. Humphrey) in a final volume of 1 ml. and incubated for 24 hr. at 4°. Guinea-pig serum diluted to have approximately 50 $C'H_{50}$ units/ml. was gently mixed with polystyrene latex (20 μg./ml.) at 0° for 6 hr. Most of the particles that sedimented as floccules were removed by centrifugation, and portions (1 ml.) were added to the sample–polystyrene latex mixtures. Complement with various amounts of polystyrene latex without sample and complement with veronal-buffered saline alone were also included as controls. After incubation for 16 hr. at 4°, the residual haemolytic activity was assayed by using sheep erythrocytes sensitized with rabbit haemolysin, by the method described by Mayer (1961). Dried guinea-pig serum as complement source, sheep blood, and rabbit anti-sheep haemolysin were obtained from Burroughs Wellcome and Co., London N.W.1.

When complement (10–50 $C'H_{50}$) was incubated directly with polystyrene latex there was an increase of the haemolytic activity as much as 15–18% relative to complement incubated in veronal-buffered saline alone, which did not vary significantly over the range of polystyrene latex used. Such activation, or probably inhibition of decay, of complement was abolished if the polystyrene latex had been coated with other proteins. After the pre-treatment of complement as described above there was no undesirable side effect of polystyrene latex.

RESULTS

Papain digestion of rabbit IgG under various conditions

Digestion at neutral pH *without reduction of labile disulphide bridges.* Lying between the compact hydrodynamic units, a pair of Fab fragments and one Fc fragment, of rabbit IgG is a region called the 'hinge' (Feinstein & Rowe, 1965) where heavy chains are presumably extended (Noelken, Nelson, Buckley & Tanford, 1965) and the labile inter-heavy-chain disulphide bridges reside. Brief papain digestion (10–20min.) at pH 7·5 without reduction of the labile disulphide bridges attacked only at the 'hinge' region (see Smyth & Utsumi, 1967). This resulted in the formation of a pre-split 7s molecule, a product analogous to that obtained with water-insoluble papain (Cebra, Givol, Silman & Katchalski, 1961), which was degraded into Fab and Fc fragments by reduction with 14 mM-2-mercaptoethanol (Figs. 1*a* and 1*b*). The Fc fragment was not dissociable at pH 2·35 but became so after mild reduction with 14mM-2-mercaptoethanol, indicating the presence of an interchain disulphide bridge (Inman & Nisonoff, 1966). This type of fragment was designated fragment lFc (Table 1).

When the reduction of the pre-split molecule was followed by alkylation with iodoacetic acid, the Fc and Fab fragments isolated contained 1·9 and 1·4 SCMC groups/mol. respectively. Since 0·2 SCMC groups/mol. were found in the light chain, and can reasonably be attributed to the light–heavy interchain disulphide bridge, the number of SCMC groups formed in Fab fragment as the result of reduction of disulphide bridges other than the light–heavy-chain bridge can be calculated as $1{\cdot}4-(0{\cdot}2\times 2)=1{\cdot}0$ which matches the 1 SCMC group/chain of Fc fragment. The Fc fragment, though it was lFc fragment, no longer formed the interchain disulphide bridge. The result seems to be consistent with the view that the pre-split 7s molecule is a product in which peptide-bond cleavage has taken place between two half-cystine residues whose disulphide bridge still links a molecule of Fab fragment with one of the two chains of Fc fragment (see Porter, 1967; Utsumi & Karush, 1965).

The formation of mFc fragment (below) was restricted by the presence of the inter-heavy-chain disulphide bridges, and even after digestion for 24hr. the product was still a mixture of lFc and mFc fragments in a ratio of 7:3 (Fig. 2*a*).

Digestion at neutral pH *with reduction of labile disulphide bridges.* When mildly reduced and alkylated IgG was digested for 10min. at pH 7·5 most of the protein was degraded into 3s fragments without forming the pre-split molecule, though only a small amount of SCMC was released in peptides. The Fc and Fab fragments contained again 2·0 and 1·0 (net) SCMC groups/mol. respectively, and the Fc fragment was identical with the lFc fragment that was obtained from the reduced alkylated pre-split molecule above.

When digestion of the reduced alkylated IgG was prolonged to 24hr., 82–86% of the initial protein-bound SCMC was released in peptides, and not more than 0·3 SCMC groups/mol. of Fc or Fab fragment remained. Most (90%) of the Fc fragment, here mFc fragment, had thus lost the interchain disulphide bridge as judged by dissociation at pH 2·35, and was distinct from lFc fragment in DEAE-cellulose chromatography (Fig. 2) and in starch-gel electrophoresis, where it was more basic than lFc fragment, although it had mol. wt. 46000, very close to that of lFc fragment (48000) (Table 1).

Digestion of mildly reduced alkylated lFc fragment with papain at pH 7·5 for 6hr. also produced mFc fragment with a concomitant release of SCMC-containing peptides. One of the peptides contained hexosamine, SCMC, threonine, serine, glutamic acid, proline and lysine in molar proportions 0·55 (uncorrected):0·90:0·87:0·77:1·00:3·55:0·84, and the other 0:0·68:0·32:0·22:1·00:2·67:0·24. The compositions are almost the same as those of the peptides obtained from the 'hinge' region of whole IgG or peptic Fab' fragment by papain digestion (Smyth & Utsumi, 1967).

Formation of sFc fragment. Papain digestion of mildly reduced alkylated IgG at pH 5·0 yielded a fragment, sFc, that was unique in serological behaviour, as described below, and more acidic than lFc fragment on electrophoresis or DEAE-cellulose chromatography (Table 1). The difference in size between sFc fragment (mol. wt. 40000) and mFc fragment was small but sufficient to separate them on gel-filtration if a long column of Sephadex G-75 was used (see the Materials and Methods section).

After being purified by the procedures described in the Materials and Methods section, the overall mass recovery of sFc fragment was 17–22% of the mass of IgG digested, corresponding to 65–87% of the theoretical mass recovery (26·7% would represent 100% recovery on the basis of mol. wt. 40000 for sFc fragment). Hence the formation of sFc fragment was attributed to further breakdown of Fc fragment rather than to an abnormal fragment derived from a small population in the heterogeneous IgG.

Further treatment of mFc fragment or reduced alkylated lFc fragment with papain at pH 5·0 also gave rise to sFc fragment, though the yield was relatively lower.

Formation of stFc fragment. Further breakdown of Fc occurred on papain digestion at pH 4·5. The product here, stFc fragment (Table 1), was analogous to, if not entirely identical with, fragment

Pep-III′ (Utsumi & Karush, 1965), a product from peptic digestion at the same pH, in mol. wt. (25 000), sedimentation coefficient (2·4s), antigenic property (as described below), electrophoretic behaviour (Fig. 7) and amino acid composition (Table 5). Hence the peptic Pep-III′ fragment was regarded as being identical with stFc fragment for the present study.

All the larger Fc fragments, lFc, mFc and sFc, could be converted into stFc fragment by further digestion with papain at pH 4·5.

Biological activities of Fc fragments

Antigenic properties of Fc fragments. From the work of Utsumi & Karush (1965), the antigenic determinants of Fc are known to be divided between two portions of the molecule: the stFc region and the region other than stFc. Since the stFc region has been shown to consist of the *C*-terminal half of the Fc fragment, including the *C*-terminal glycine residue (Prahl, 1967), these groups of determinants

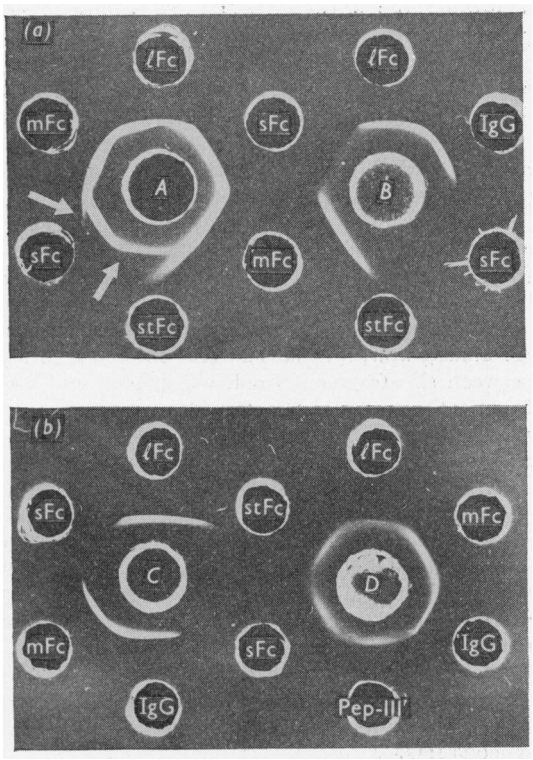

Fig. 3. Precipitin reactions of Fc fragments in gel. (*a*) Four types of Fc fragments (lFc, mFc, sFc and stFc) and intact IgG placed in the peripheral wells were treated with (*A*) anti-(lFc fragment) and *B*, anti-(lFc fragment) absorbed with stFc in the centre wells. Arrows indicate faint spurs. (*b*) The same antigens as those in (*a*), and Pep-III′ (Utsumi & Karush, 1965) (stFc fragment derived from peptic digestion of IgG), were reacted with (*C*) anti-(sFc fragment) absorbed with stFc fragment and (*D*) anti-(stFc fragment).

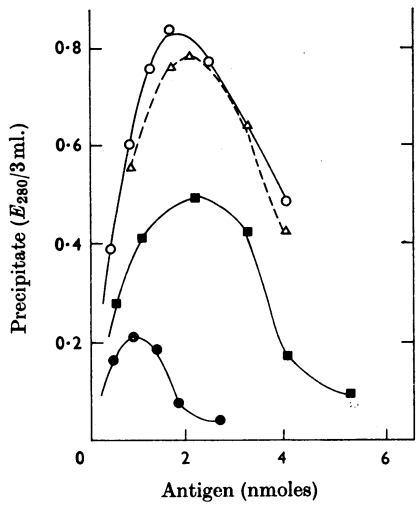

Fig. 4. Precipitin curves of goat anti-(lFc fragment) with IgG (○), lFc fragment (△), sFc fragment (■), and stFc fragment (●). Precipitates formed with 0·2 ml. of the antiserum were dissolved in 3·0 ml. of 0·1 M-sodium decyl sulphate, pH 8, and the E_{280} was measured. The behaviour of mFc fragment was almost identical with that of lFc fragment.

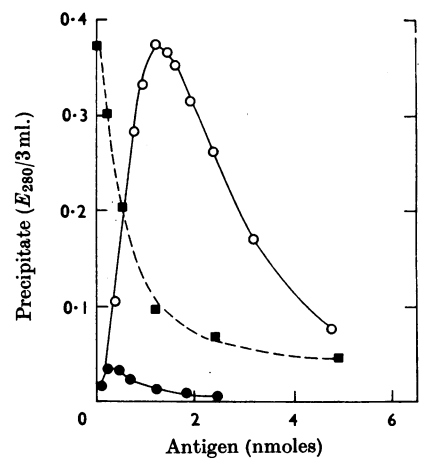

Fig. 5. Precipitin reactions of lFc fragment (○) and sFc fragment (●) with anti-(lFc fragment) absorbed with stFc, and inhibition of the precipitin reaction of lFc fragment by sFc fragment (■). The procedures used were the same as those in Fig. 4. For the inhibition experiment, increasing amounts of sFc fragment were added to the equivalence mixture of lFc fragment (1·2 nmoles) and the absorbed antiserum (0·2 ml.) in a final volume of 0·4 ml.

are referred to as the *C*-terminal and *N*-terminal determinants, respectively. Goat antiserum to lFc fragment contained antibodies to both the groups of determinants, and absorption of it with stFc fragment gave antiserum specific for the *N*-terminal determinants alone (Figs. 3, 4 and 5), whereas immunization of the goat with stFc fragment produced antiserum specific to the *C*-terminal group, anti-(stFc fragment).

The reactions in gel of the Fc fragments with these antisera are illustrated in Fig. 3, and some of the results of quantitative precipitin reactions in Figs. 4 and 5. As far as was tested, mFc fragment showed no antigenic deficiency relative to lFc fragment. All the Fc fragments, lFc, mFc, sFc, and stFc from both papain and peptic digestion, and intact IgG reacted identically with anti-(stFc fragment) (Fig. 3*D*) and gave the same antibody/antigen molar ratio of 2:1 at equivalence in the quantitative precipitin reactions. A similar ratio was also obtained by the reaction of stFc fragment with anti-(lFc fragment) (Fig. 4). Therefore the antigenic structure of the *C*-terminal portion of Fc fragment is retained completely by all these fragments.

In the reaction with antiserum to the *N*-terminal determinants [anti-(lFc fragment) absorbed with stFc fragment], sFc fragment gave only a very small amount of precipitate (Figs. 3*B* and 5). In apparent conflict with this result is the behaviour of sFc fragment in the reaction with unabsorbed anti-lFc fragment, where it precipitated approx. 50% of antibody to the *N*-terminal determinants (compare sFc fragment with stFc fragment and lFc fragment in Fig. 4) and only a slight spur, if any, was seen between sFc fragment and lFc fragment or mFc fragment in gel (Fig. 3*A*). This is by no means accounted for by the small amount of precipitate given by the absorbed serum. On the other hand, sFc fragment was found to be strongly inhibitory to the precipitin reaction of lFc fragment with antibodies to the *N*-terminal determinants (Fig. 5). The inhibition reached 50% and 75% at molar ratios (sFc fragment/lFc fragment) 0·5 and 1 respectively, and further increase of sFc fragment abolished the precipitation of lFc fragment completely. In the presence of antibodies to both groups of antigenic determinants the precipitin reaction due to the intact *C*-terminal determinants of sFc fragment would cause a partial co-precipitation of antibodies to the *N*-terminal determinants.

Anti-(sFc fragment) obtained by immunization of the goat with pure sFc fragment gave a pattern of precipitin reactions with these fragments very similar to that obtained with anti-(lFc fragment). After being absorbed with stFc fragment, it no longer precipitated with sFc fragment (Fig. 3*C*) but retained antibodies whose precipitin reaction with lFc fragment was again quantitatively inhibited by sFc fragment.

Passive cutaneous anaphylaxis. A striking difference between fragments lFc and mFc was revealed in the capacity to sensitize guinea pigs for reverse passive cutaneous anaphylaxis (Table 2). Of the seven preparations of mFc fragment tested, all but one gave negative reactions at a concentration of 10 μg./ml., whereas lFc fragment and intact IgG induced positive reactions at 1 μg./ml. without exception. The shorter fragments, sFc and stFc, were completely inactive.

To test whether the inactivity of mFc fragment is due to its lack of fixation to skin, a mixture of intact IgG and mFc fragment at final concentrations of 0·5 μg. and 20 μg./ml. respectively was injected intracutaneously, and the passive cutaneous anaphylaxis was provoked by non-cross-reacting goat anti-(Fab fragment). Fragment lFc at the same ratio markedly inhibited the reaction of IgG, but no effect of mFc fragment was obtained.

Complement fixation. As shown in Fig. 6, lFc fragment and mFc fragment retained at least a part of the capacity of IgG to bind guinea-pig complement, but fragments sFc and stFc lost the activity completely.

Table 2. *Reverse passive cutaneous anaphylaxis tests with Fc fragments*

Guinea pigs were injected intracutaneously with samples (0·1 ml.) of the fragments at various concentrations, and after 4 hr. intravenously with 0·3 ml. of goat anti-(stFc fragment) serum mixed with 0·3 ml. of 1% Evans Blue. Reactions giving areas of 4 mm. diameter or larger were scored positive. The results are expressed as no. of positive reactions/no. of total test sites.

Concn. (μg./ml.) Fragment ...	Positive reactions				
	IgG	lFc	mFc	sFc	stFc
100	—	3/3	6/22	0/12	0/12
10	20/20	14/14	2/30	0/24	0/8
5	—	8/8	0/14	0/4	0/4
1	60/60	15/15	0/28	0/4	—
0·5	12/12	15/16	0/7	—	—
0·2	10/12	6/16	—	—	—
0·1	2/6	0/6	—	—	—

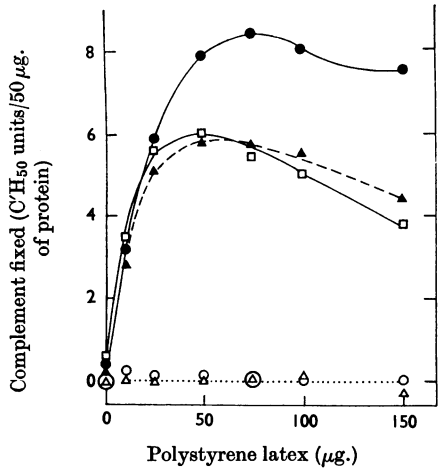

Fig. 6. Complement-fixation by intact IgG (●), lFc fragment (□), mFc fragment (▲), sFc fragment (○), and stFc fragment (△). The results are expressed by the amount of complement ($C'H_{50}$ units) fixed by 50 μg. of protein as a function of the amount of polystyrene latex added. For details of the method, see the Materials and Methods section.

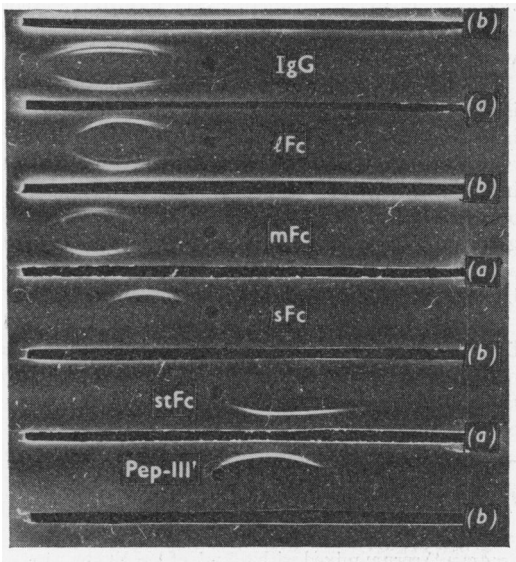

Fig. 7. Immunoelectrophoretic pattern of rabbit IgG and its fragments, lFc, mFc, sFc, stFc and Pep-III′ (Utsumi & Karush, 1965) (stFc from peptic digestion). In the troughs were (*a*) anti-(stFc fragment) and (*b*) anti-(lFc fragment) absorbed with stFc fragment.

Characterization of Fc fragments

Electrophoretic behaviour. The behaviour of the Fc fragments on gel electrophoresis grossly paralleled that in DEAE-chromatography (Table 1).

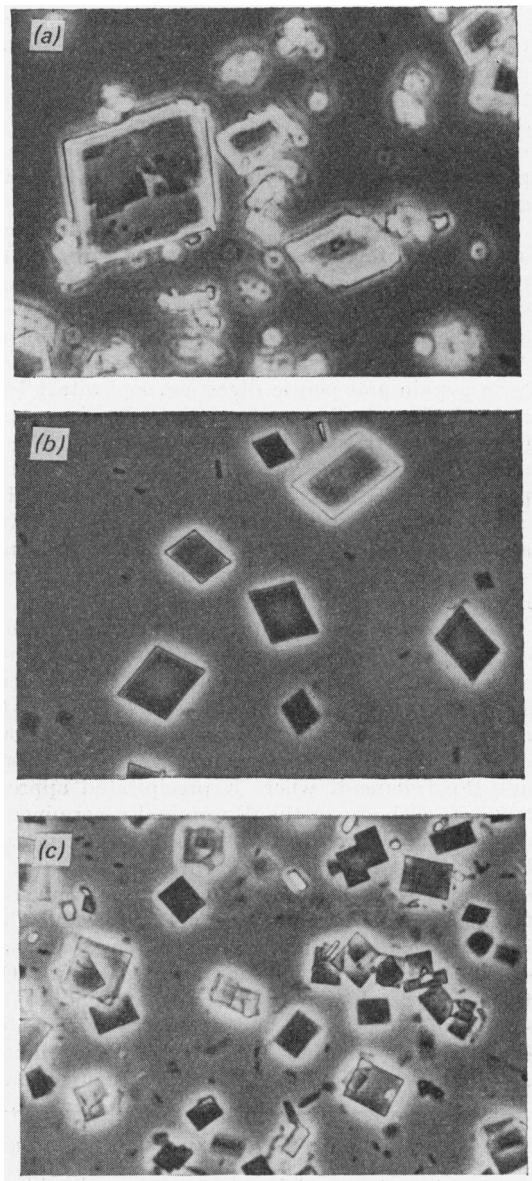

Fig. 8. Crystals of fragments lFc (*a*), mFc (*b*), and stFc (*c*); magnification × 624.

Under the conditions described in the Materials and Methods section, stFc fragment migrated the fastest towards the anode, followed by fragments sFc, lFc and mFc, in that order. The relative mobility of each fragment with its characteristic antigenic activity is illustrated by immunoelectrophoresis in Fig. 7.

Crystallization. The crystals of fragment lFc grew to large, rather irregular rhomboids, whereas

those of fragment mFc were elongated rhomboids of more regular contour, and the crystals of fragment stFc that were formed by freezing slowly at neutral pH were rectangular and small but thick (Fig. 8). In marked contrast, fragment sFc was highly soluble and did not crystallize under any of the conditions tested.

Carbohydrate content. So far as is known, there is only one carbohydrate moiety in the Fc region of rabbit IgG, C_2, which contains 4 hexosamine and 3–4 hexose residues/mol. (Nolan & Smith, 1962). Another carbohydrate group of rabbit IgG, C_1, contains only 1 hexosamine and a few hexose residues/mol. (Utsumi & Karush, 1965) and has been shown to be present in the 'hinge' region (Smyth & Utsumi, 1967). That the minor group, C_1, is present in the *N*-terminal region of lFc fragment, at least in some of the molecules, but is absent from mFc fragment was suggested by the release of a peptide carrying 1 residue of hexosamine/mol. on conversion of fragment lFc into fragment mFc by papain as described above. On the other hand, the relatively high content of both hexosamine and hexose found in both mFc fragment (3·3 hexosamine and 3·1 hexose residues/chain, uncorrected for losses) and sFc fragment (3·3 hexosamine and 2·9 hexose residues/chain) suggested that these fragments as well as fragment lFc carried the carbohydrate group C_2. Fragment stFc did not contain any carbohydrate.

Disulphide bridges in Fc fragments. The results of thiol titration with 5,5′-dithiobis(2-nitrobenzoic acid) after limited or extensive reduction are summarized in Table 3. Fragment lFc possessed a single labile disulphide bridge, but fragment mFc was devoid of it, in accordance with the absence of the interchain bond, but both fragments contained two resistant disulphide bridges. The two thiol groups/chain of stFc fragment are attributed to a single pair of intrachain disulphide bridges in this region of the molecule (Prahl, 1967). A relatively large number of free thiol groups detectable in sFc fragment may not be due to a labile disulphide bridge, because the number of reactive thiol groups did not significantly increase after mild reduction. Of the total three thiol groups/chain of sFc fragment, one was lost by the treatment with papain at pH 4·5 that converted sFc fragment into stFc fragment (Table 3).

Table 3. *Disulphide bridges of Fc fragments*

Samples reduced with 4mM-2-mercaptoethanol for 2hr. at pH 8·0 (limited reduction), or with 0·56M-2-mercaptoethanol in the presence of 7M-guanidine–HCl for 5hr. at pH 8·0 (extensive reduction), and unreduced proteins were precipitated and washed with 5% trichloroacetic acid, and thiol groups were titrated with 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of 0·1M-sodium decyl sulphate.

	Thiol groups (moles/mole of half-Fc)		
Fragment	Unreduced	Limited reduction	Extensive reduction
lFc	0·04	0·70	4·85
mFc	0·06	0·09	4·10
sFc	0·30	0·41*	2·95
stFc	0·02	0·64†	2·04
stFc from sFc‡	0·04	—	2·08

*Average of values varying from 0·20 to 0·67.

†The extinction in this reaction developed only slowly and the value listed was taken after 60min. incubation with 5,5′-dithiobis(2-nitrobenzoic acid).

‡Fragment sFc was further treated with papain at pH 4·5 in the presence of 14mM-2-mercaptoethanol, and the stFc fragment formed was isolated by gel filtration on Sephadex G-75.

DISCUSSION

In the present study the formation of four types of Fc fragments (see Fig. 9) with different sizes by papain took a stepwise course. Larger fragments were thus converted into smaller ones by further digestion under appropriate conditions. Papain first attacked at the 'hinge' region of intact rabbit IgG to give lFc fragment, but further breakdown of Fc appeared to be related to a series of changes in the quaternary structure that are effected by reduction of the inter-heavy-chain disulphide bridges to give mFc fragment, and by lowering the pH to give sFc and stFc fragments. Of particular note in this connexion is the observation that the conformation of the Fc portion of rabbit IgG changes continuously as the pH is lowered, but the dissociability at pH 2·4 of fragments mFc, sFc and stFc decreases in this order (Charlwood & Utsumi, 1969). The failure of papain to attack the *C*-terminal stFc region of Fc even at pH 4·5 seems to be due mainly to the stability of the quaternary structure of this region, whereas the *N*-terminal structure, if the inter-heavy-chain disulphide bridges are absent, is relatively unstable and thus susceptible to the enzyme. The larger fragments, lFc, mFc and sFc, which were produced under milder digestion conditions, also contained the *C*-terminal region, since all of them carried the intact antigenic structure of stFc fragment and were converted into stFc fragment by the action of papain at pH 4·5. None of the Fc fragments gave any significant release of amino acid residues with carboxypeptidases (S. Utsumi, unpublished work), probably due to the -Pro-Gly sequence at the *C*-terminal end of heavy chain (Givol & Porter, 1965).

The presence of an interchain disulphide bridge in lFc fragment is evident because it was not dissociable at pH 2·35 but became so after reduction of a

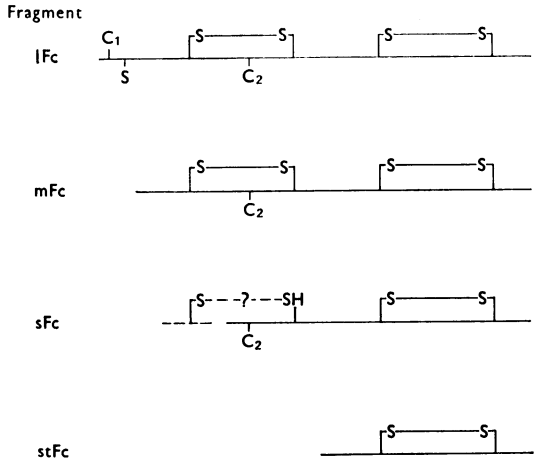

Fig. 9. Schematic models of four types of Fc fragments of rabbit IgG. Fragment lFc possesses two intrachain disulphide bridges, the carbohydrate group C_2, a single interchain disulphide bridge, and, in some of the molecules, the minor carbohydrate group C_1, whose hexosamine residue was shown to be associated with one of the 'hinge' peptides liberated from lFc fragment (cf. Smyth & Utsumi, 1967). Fragment mFc has lost the 'hinge' region, the *N*-terminal portion of lFc fragment, though it still contains the two interchain disulphides and carbohydrate C_2. Fragment sFc retains the *C*-terminal loop with an intrachain disulphide bridge and carbohydrate C_2, but has lost one of the half-cystine residues, presumably from the *N*-terminal intrachain disulphide bridge. Fragment stFc has been shown to consist of about 110 amino acid residues/chain, corresponding to the *C*-terminal half of Fc fragment.

Table 4. *Amino acid composition of lFc and mFc fragments*

Analyses were made of 24hr. hydrolysates, and the composition was compared with that of the Fc with 216 amino acid residues/chain analysed by Hill *et al.* (1967). The SCMC of lFc fragment was formed on reduction and alkylation of the pre-split molecule. Tryptophan was not measured in fragments lFc and mFc.

	Amino acid composition (moles of amino acid/moles of half-Fc)		
Fragment ...	lFc	mFc	Fc (216 residues) (Hill *et al.* 1967)
SCMC	1·0	—	—
Aspartic acid	18·0	18·0	18
Threonine	16·0	14·2	14
Serine	20·0	18·7	20
Glutamic acid	24·8	23·8	25
Proline	22·8	19·6	23
Glycine	10·5	10·5	11
Alanine	8·6	8·5	8
Half-cystine	3·2	3·5	5
Valine	17·4	17·1	17
Methionine	3·4	3·4	4
Isoleucine	10·0	10·0	9
Leucine	13·7	13·0	14
Tyrosine	6·8	6·8	7
Phenylalanine	8·0	7·9	8
Lysine	14·3	13·0	13
Histidine	4·8	4·8	5
Arginine	11·0	11·3	11
Tryptophan	—	—	4

single labile disulphide bridge (Table 3). Prolonged papain digestion of lFc fragment that had been reduced and alkylated gave mFc fragment and released 'hinge' peptides (Smyth & Utsumi, 1967), one of which corresponded to the region -Ser-Lys-Pro-Thr-SCMC-Pro-Pro-Pro-Glu- (Cebra, Steiner & Porter, 1968) and the other to an overlapping region of -SCMC-Pro-Pro-Pro-Glu-, the *N*-terminal end of the Fc fragment according to Hill, Lebovitz, Fellows & Delaney (1967). Although the question of whether any other peptides were also released stoicheiometrically from the fragment lFc molecule was not examined, the difference in the molecular weight (Table 1) between fragments lFc and mFc, 1000 per chain, corresponded to the average size of the 'hinge' peptides. Further, as shown in Table 4, the loss of amino acids in fragment mFc compared with fragment lFc, one glutamic acid residue, three proline residues and one leucine residue and probably some threonine and serine, agrees fairly well with the composition of the larger SCMC peptide. It may therefore be inferred that the portion of the 'hinge' region including the interchain disulphide bridge is located at the *N*-terminal end of fragment lFc and that papain gradually removes this region to form fragment mFc. From the preliminary assay by the dinitrophenylation method, leucine alone (0·6 mol./chain) was detected as the *N*-terminal residue of fragment mFc. The present results, however, are not sufficient to rule out the possibility that papain cleavage might also have occurred elsewhere in the fragment mFc molecule with only a small number of amino acids released, in such a manner that it resulted in two portions that were still held together by a disulphide bridge. However, such a peptide break would not give the quantitative recovery of protein carrying four thiol groups/chain (Table 3), which represent both the intrachain disulphide bridges of Fc, after extensive reduction and subsequent precipitation with trichloroacetic acid.

On the other hand, this may indeed be the case for sFc fragment, which contained three thiol groups/chain after extensive reduction. If two thiol groups are attributed to the disulphide bridge in the region of fragment stFc (Table 3), the remaining one must be derived from the *N*-terminal intrachain disulphide of Fc (Fig. 9), since the native

Fc molecule contains two intrachain disulphide bridges but no free thiol group (Hill *et al.* 1967; Frangione, Milstein & Franklin, 1968). The third thiol group of reduced sFc fragment cannot be derived from the interchain disulphide of lFc fragment, since sFc fragment is the further breakdown product of mFc fragment which has already lost this region. That most, if not all, of the fragment sFc molecules carried the pair of carbohydrate groups of Fc (C_2 in Fig. 9) is clear from the carbohydrate content of the fragment [4·1 hexosamine residues and 3·2 hexose residues/chain after correction for the losses during hydrolysis and Dowex treatment (19% and 9% respectively; Utsumi & Karush, 1965)], which is almost the same as that of C_2 (Nolan & Smith, 1962). After extensive reduction, the carbohydrate group was still associated with sFc protein. In view of the locations of C_2 (position 150, counted from the *C*-terminus) and the two half-cystine residues forming the *N*-terminal loop of disulphide (positions 126 and 180), as determined by Hill *et al.* (1967), it is inferred that the peptide cleavage took place somewhere to the *N*-terminal side of C_2, and that the apparent free thiol group of sFc was derived from residue 126, rather than 180 (Fig. 9). However, more chemical analyses are needed for final clarification.

Since the digestion took place in the absence of reducing agent, how such an anomalous product as sFc fragment could occur is also unknown. A possibility would be that a small portion containing residue 180 (Fig. 9) was still covalently attached to the sFc molecule. Attempts to identify such a peptide, which would be released by reduction of sFc fragment, were, however, unsuccessful. Alternatively, the small portion might have been removed by reduction of the disulphide during the purification procedures with CM- and DEAE-cellulose. In any case, the amino acid composition of fragment sFc (mol. wt. 40000) appeared to match fairly well with that of the first 167 amino acid residues from the *C*-terminus of Fc (Table 5). The presence of the pair of large carbohydrate groups (C_2) near the *N*-terminal end of sFc fragment explains its high solubility and non-crystallizability.

The amino acid composition of a preparation of stFc fragment is also shown in Table 5; it is almost identical with that of the first 109 residues from the *C*-terminus of the sequence found by Hill *et al.* (1967).

The observed identity in the antigenic activity of fragments lFc and mFc is consistent with the lack of antigenic structure in the 'hinge' region of rabbit IgG, as has been suggested previously (Jaquet & Cebra, 1965; Utsumi & Karush, 1965). The identical reactivity of fragment stFc and intact IgG, as well as of all the larger Fc fragments, with antiserum to fragment stFc suggests that fragment

Table 5. *Amino acid composition of sFc and stFc fragments*

Analysis of sFc fragment was made at a single hydrolysis time of 24 hr., and the composition of stFc fragment was determined from analyses of an extensively reduced alkylated sample, after hydrolysis for 22 and 44 hr. For comparison, the amino acid composition of the first 167 and the first 109 residues from the *C*-terminus of Fc was calculated from the amino acid sequence given by Hill *et al.* (1967). Tryptophan was not measured in fragments sFc and stFc.

	Amino acid composition (moles of amino acid/mole of half-Fc)			
Fragment ...	sFc	First 167 residues of Fc (Hill *et al.* 1967)	stFc	First 109 residues of Fc (Hill *et al.* 1967)
Aspartic acid	13·1	13	9·0	9
Threonine	11·0	12	6·7	7
Serine	16·8	17	14·4	15
Glutamic acid	18·1	18	10·2	10
Proline	15·0	15	7·9	8
Glycine	9·2	9	7·4	8
Alanine	6·7	8	3·8	4
Half-cystine	1·6	3	2·0	2
Valine	12·2	12	6·9	7
Methionine	3·0	3	2·6	3
Isoleucine	5·9	6	3·2	3
Leucine	9·7	11	6·8	7
Tyrosine	6·5	5	5·2	5
Phenylalanine	5·6	5	2·9	3
Lysine	11·0	11	6·4	7
Histidine	5·0	5	2·9	3
Arginine	9·8	10	4·8	5
Tryptophan	—	4	—	3

stFc may truly represent the configuration of the corresponding region of the native IgG molecule.

Although the results indicated that fragment sFc retained most, if not all, of the *N*-terminal determinants, being fully reactive with antibody and normally immunogenic to goats, the fragment was curiously incapable of precipitating with antibody. Perhaps the loss of some of the *N*-terminal structure of the Fc molecule allows bivalent binding of a single antibody to a pair of homologous determinants in sFc fragment. In lFc and mFc fragments steric constraints may prohibit this bivalent reaction, thus forcing antibody to cross-link the antigen molecules.

Fragment mFc, which differs from fragment lFc probably only by the 'hinge' region, has lost the activity to sensitize guinea-pig skin almost completely. The inconsistently observed positive reactions at high protein concentration may best be accounted for by a small amount of contamination of fragment mFc by fragment lFc (less than 1%) because even at a concentration as high as 100 μg./ml. the reaction, when given, was small and well localized, whereas fragment lFc at this concentration produced a large, diffuse reaction. Although the results strongly suggest an essential role of the 'hinge' region in the fixation of IgG to the guinea-pig skin sites, no passive cutaneous anaphylaxis, reverse or direct, is given by the peptic divalent $F(ab')_2$ fragment (Ovary & Taranta, 1963), which has been shown to contain the 'hinge' region at its *C*-terminus (Smyth & Utsumi, 1967). Whether this is to be attributed to some alteration of the structure of the 'hinge' region in this molecule, or to the loss of complement-binding activity, remains to be clarified.

The present new assay method for complement-fixation using protein layers on the surface of polystyrene latex seems to be more quantitative than other methods with physically or chemically formed aggregates (Ishizaka & Ishizaka, 1960). The specificity of the complement-fixation in the present system was verified with various proteins that are known to fix or not to fix guinea-pig complement, including guinea pig γG_2- and γG_1-immunoglobulins (Bloch, Kourilsky, Ovary & Benacerraf, 1963) (the samples were generously provided by Dr J. H. Humphrey). The guinea pig γG_2-immunoglobulin preparation fixed 38×10^3 $C'H_{50}$ units/μmole, whereas no fixation was detected with the γG_2-immunoglobulin preparation in this system. In spite of the presence of such a strong complement-fixer as γG_2-immunoglobulin in the complement source (serum) there was no loss of complement due to this. This may be simply attributed to a mixed aggregation of proteins in which close proximity of γG_2-immunoglobulin molecules rarely happens.

There was no change in complement-binding activity due to the removal of the 'hinge' region, since both lFc and mFc fragments fixed the same amount of complement, 6×10^3 $C'H_{50}$ units/μmole. If it is assumed that intact IgG and Fc molecules adsorbed on the latex surface had the same efficiency for complement-binding, both Fc fragments appeared to be considerably less active than intact IgG, which fixed 25×10^3 $C'H_{50}$ units/μmole. Even if the technical ambiguity could be eliminated, it would be too early to speculate whether this is due to a difference in affinity or to the presence of two combining sites on the IgG molecule for two complement components, compared with one on the Fc fragments.

As has been described by Prahl (1967), the *C*-terminal region of Fc (the stFc region) does not carry any of the biological activities except the species-specific antigenic structure. In view of the strong non-covalent interactions between the chains in stFc fragment (Charlwood & Utsumi, 1969), however, the important role of this region would be that of stabilizing the quaternary structure of Fc fragment. The sFc portion too is a major antigenic site but lacks tissue- (guinea-pig skin-) as well as complement-binding sites. Thus these two typical biological functions other than the specific antigen binding so far appear to be exhibited by a region of some 50 amino acid residues/chain: the guinea-pig skin fixation is principally associated with the 'hinge' region, and the complement-binding site with the region present in mFc fragment but missing from sFc fragment. However, an alternative possibility still remains that these activities are effected by the co-operation of much wider areas than the limited *N*-terminal region of the molecule.

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