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Characterization of a Plasmin-Digest Fragment of Rabbit Immunoglobulin Gamma that binds Antigen and Complement

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Rabbit immunoglobulin gamma (IgG) was digested with plasmin after being left for 15min at pH 2.5, 30°C followed by a rapid increase in the pH to 7. The fragment antigen and complement binding (Facb) was isolated and characterized chemically and biologically. Sequence studies showed that the *C*-terminal quarter of the heavy chain had been removed, the split occurring at a lysine–alanine bond in the sequence Thr-Ile-Ser-Lys-Ala-Arg. The fragment Facb retained the capacity to precipitate with antigen and the precipitate caused activation of the first component of complement of the same order as that of acid-treated IgG. Both Facb and acid-treated IgG showed a fall in complement fixation relative to the native molecule of 30–40%.

Since the description of Facb (Fragment Antigen and Complement Binding), a fragment formed by the cleavage of rabbit IgG† by plasmin (Connell & Porter, 1971), several reports have compared its biological properties with other proteolytic fragments of IgG, Fab, (Fab′)$_2$, Fc and pFc′ (Stewart *et al.*, 1973; MacLennan *et al.*, 1974). Fig. 1 shows the relationship of these split products to each other and to the whole molecule. The results emphasize the specific role of the C_H2 domain in the binding and activation of the first component of complement C1. By preserving the relationship of the antigen-binding sites and the C_H2 domain, Facb appears to offer a simplified approach to the study of the binding and activation of C1. This has advantages over methods causing more extensive destruction of IgG with loss of complement-binding properties (Utsumi, 1969; Kehoe & Fougereau, 1969; Ellerson *et al.*, 1972). The smaller fragment (Fab′)$_2$ was shown by Reid (1971) to retain the capacity to fix complement via the alternative pathway but not to activate C1 and the subsequent components by the classical pathway.

The preparation and properties of Facb were therefore investigated more fully, the point of hydrolysis by plasmin was determined and the chemical characteristics and complement-fixing ability of the fragment were studied. The preliminary findings of Connell & Porter (1971) have been confirmed and extended.

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† Abbreviations: IgG, immunoglobulin gamma; Fab fragment, *N*-terminal half of heavy chain and light chain; (Fab′)$_2$, *N*-terminal half of heavy chain and light chains joined by inter-heavy-chain disulphide bond; Fc, *C*-terminal half of heavy-chain dimer; pFc′, *C*-terminal quarter of heavy-chain dimer. These fragments are shown in Fig. 1(*a*). V_H, C_H1, C_H2 and C_H3 are homology regions of the heavy chain and are shown in Fig. 1(*b*). C1, the first component of complement; EAC′4, sheep erythrocytes coated with heated rabbit anti-(sheep erythrocytes) together with the fourth component of complement.

Materials and Methods

Purification of rabbit IgG

This was purified from pooled rabbit serum as described by Prahl & Porter (1968); after purification with Na_2SO_4 the IgG was fractionated on a column of DEAE-Sephadex A-50 equilibrated with 0.07M-sodium phosphate buffer, pH 6.3. Different cuts of the IgG eluted under these conditions were chosen according to the associated intrinsic proteolytic activity. Detection of the protease–esterase activity with *N*-α-benzoyl-L-arginine ethyl ester as substrate (Schwert & Takenaka, 1955) was used for the screening of the DEAE-Sephadex eluate. The early IgG fractions eluted from DEAE-Sephadex had less than 0.08nkat of proteolytic activity/mg at 25°C and were used for all experiments. After dialysis against 4mM-sodium acetate buffer, pH 5.4, to minimize aggregation (Stevenson & Dorrington, 1970), the IgG fractions were ultrafiltered on Amicon PM10 membranes (Amicon, High Wycombe, Bucks., U.K.) to a concentration of 20mg/ml and rapidly frozen.

Plasmin

Human plasmin purchased from Kabi Pharmaceuticals (Uxbridge Road, Ealing, London, U.K.) was used for the preparation of Facb. The freeze-dried powder was dissolved either in aq. 50% (v/v) glycerol or in water to a concentration of 1.6mg/ml, corresponding to 67nkat/ml with *N*-α-benzoyl-L-arginine

ethyl ester as substrate at 25°C (25 caseinolytic units/ml). No attempt was made to remove the salts or the remaining lysine in the commercial product.

CNBr cleavage of fragment Facb

CNBr cleavage of Facb to isolate the C-3 fragment was done at 4°C for 24h with a CNBr/protein weight ratio of 2:1, in 70% (v/v) formic acid (Givol & Porter, 1965).

Tryptic digestion of the C-3 fragment

This was performed with bovine trypsin (Worthington Co., Freehold, N.J., U.S.A.) treated with 1-chloro-4-phenyl-3-L-tosyl-*p*-sulphonamidobutan-2-one at 37°C for 3h with an enzyme/substrate protein ratio of 1:50, at a concentration of 10mg of C-3 peptide/ml in 0.2M-*N*-ethyl morpholine–acetic acid buffer, pH 8.5. The incubation medium was then quickly frozen and freeze-dried: the tryptic peptides were solubilized in 50mM-NH_3 for further studies.

The tryptic peptides from C-3 were separated by high-voltage electrophoresis at pH 3.5, 6.5 and (or) 1.9. They were located first with a collidine–ninhydrin spray; arginine-containing peptides were detected by their fluorescence as described by Yamada & Itano (1966); histidine peptides were detected by the Pauly reaction and tyrosine peptides with α-nitroso-β-naphthol reagent (Hopkin and Williams, Chadwell Heath, Essex, U.K.).

The peptides separated by electrophoresis were eluted with 0.2M-acetic acid (Reid, 1974). Radioautography of the three *S*-carboxymethylcysteine-containing peptides was performed as described by O'Donnell *et al.* (1970).

Carboxypeptidase B digestion of the C-3 fragment from Facb

This was performed at 37°C at an enzyme/substrate protein ratio of 1:10 in 0.1M-sodium phosphate buffer, pH 7.4, with 0.1% (w/v) sodium dodecyl sulphate. At different times samples were withdrawn, placed in 6vol. of 0.1M-HCl and immediately cooled. The different samples were then kept frozen before being applied directly to the amino acid analyser. A portion treated with HCl before the addition of carboxypeptidase was used to measure the free amino acids in the initial substrate and in the enzyme.

N-Terminal amino acids

These were identified by the dansyl method (Gray, 1967), the dansyl-amino acid being fractionated on polyamide sheets (5cm × 5cm) as described by Woods & Wang (1967).

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

This was carried out as described by Fairbanks *et al.* (1971). When reduction of the samples was necessary they were incubated with 50mM-dithiothreitol–4M-urea–1% (w/v) sodium dodecyl sulphate at 37°C for 60min and then with 120mM-iodoacetamide for a further 30min at the same temperature. Coomassie Blue was used for protein staining (Weber & Osborn, 1969).

Complement fixation

This was measured on pre-formed immune aggregates as described by Reid (1971). The immune precipitate was incubated with undiluted guinea-pig serum for 18h at 2–4°C. After centrifugation at 5000*g* for 10min the residual activity was titrated on dilutions of the supernatant.

C1 binding was determined as described by Ishizaka *et al.* (1966) by using EAC′4 cells prepared as described by Borsos & Cooper (1961).

Anti-ovalbumin was raised in rabbits as described by Porter (1955) and purified as described for IgG. The binding assays were done on the monomeric fractions of IgG, acid-treated IgG and Facb eluted from Sephadex G-150 columns.

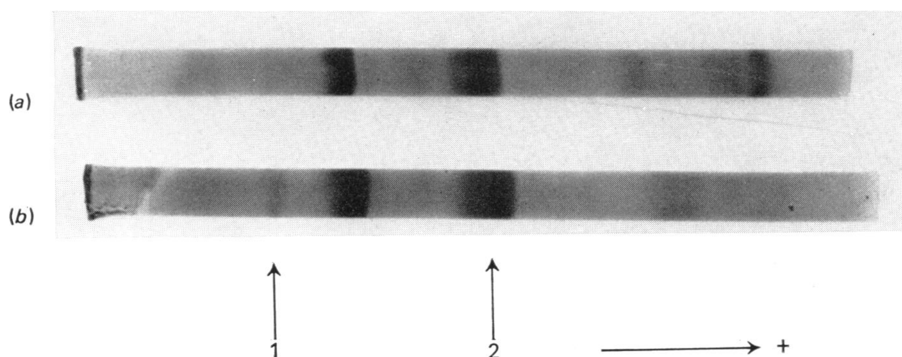
A precipitin curve was used to determine the equivalence point and the specific-antibody content of each fraction. The immune precipitate obtained at the equivalence point after incubation for 1h at 37°C, followed by 16h at 2–4°C, was washed three times with cold 0.14M-NaCl and finally suspended in the buffer used for the complement assay. Protein was determined by absorption at 280nm on portions of the suspension dissolved in 0.1M-NaOH. The ovalbumin content was deducted to calculate the amount of specific anti-ovalbumin IgG.

Results

Preparation of Facb

Pooled rabbit IgG (20mg/ml) in 4mM-sodium acetate buffer, pH 5.4, was adjusted to pH 2.5 by addition of 1M-HCl with careful mixing. After 15min of incubation at 30°C the pH was rapidly raised to 7 with 1M-NaOH and plasmin added immediately to give 0.3nkat/mg of IgG.

After incubation at 30°C for 10min, the enzymic digestion was stopped by cooling to 0°C and either (for the chemical characterization) the samples were adjusted to pH 3.3 by addition to the incubation medium of an equal volume of 12M-urea–0.4M-sodium formate, pH 3.3, or (for the purification of Facb used in biological assays) soya-bean trypsin inhibitor in 0.1M-Tris–HCl, pH 8.0, was added to the digest in a twofold excess over plasmin, on a molar basis.



EXPLANATION OF PLATE I

Plasmin-hydrolysis products electrophoresed on polyacrylamide gel in sodium dodecyl sulphate

(*a*) Whole digest. (*b*) Facb eluted from Sephadex G-150 in 6M urea–formate buffer, pH 3.3. The samples (20 μg of protein) were reduced and alkylated as described in the Materials and Methods section. Electrophoresis was for 3 h at 90 V. Arrows 1 and 2 show the positions of IgG heavy and light chains respectively.

For chemical study, the digest in urea–formate, containing about 95% of split IgG as judged from sodium dodecyl sulphate–polyacrylamide-gel electrophoresis on reduced and alkylated samples (Plate 1*a*), was applied to a column of Sephadex G-150 in 6M-urea–0.2M-sodium formate, pH3.3. Facb was recovered in the first peak eluted, with an average yield of 75% of the expected theoretical yield. Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis showed that the Facb was separated by gel filtration on Sephadex G-150 from the split products eluted in the second peak, but not from about 5% unsplit IgG (Plate 1*b*).

For complement-fixation studies the digest containing soya-bean inhibitor was applied to a column of Sephadex G-150 in 0.05M-Tris–HCl, pH7.4, and 0.14M-NaCl; only the fractions eluted at the maximum of the first peak were used for the binding assays. These also contained up to 5% unsplit IgG.

Preparation of the C-terminal CNBr fragment of Facb (Facb C-3)

The Facb fragment eluted from the Sephadex G-150 column in 6M-urea–formate buffer was dialysed against 0.2M-acetic acid at 4°C and freeze-dried. The fragment was dissolved in 98% (v/v) formic acid which was then diluted to 70% with water before addition of CNBr, as described above. After the reaction, the solution was diluted 10-fold with water and the CNBr removed by freeze-drying. The digest was taken up in 6M-urea–0.2M-sodium formate, pH3.3, and fractionated on a column of Sephadex G-100 equilibrated in the same medium. The *C*-terminal CNBr fragment (Facb C-3, by analogy with IgG CNBr fragment) was eluted in a peak well resolved from the bulk of the material. The average yield of 65–70% of the theoretical value is similar to that obtained from non-cleaved heavy chains: for subsequent studies Facb C-3 was dialysed against 0.2M-acetic acid and freeze-dried.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis on Facb C-3 at this stage showed a purity of about 95%. The contaminant observed on the gel is the IgG C-4 fragment due to the presence of a small amount of IgG remaining in Facb; this fragment cannot be separated from Facb C-3 on Sephadex G-100. When the gel is run with the reduced and alkylated Facb C-3 this contaminant appears as a single band as fragment C-4 is split into C-4B and C-4A (Fig. 1*b*). The C-4A fragment is of small size and is not detectable on the gel, but the content of the C-4B fragment gives an estimate of purity.

Characterization of the terminal amino acids of Facb C-3

All structural investigations were done by comparison with a CNBr fragment C-3 prepared from pooled rabbit IgG. The amino acid composition of the two fragments Facb C-3 and IgG C-3 is given in Table 1. The *N*-terminal amino acid of Facb C-3 was found by the dansyl technique to be isoleucine. No significant yield of other dansyl derivatives was observed on 20nmol samples. Digestion of Facb C-3 with carboxypeptidase B, as described in the Materials and Methods section, showed a selective release of lysine (Table 2), as expected from the main specificity of plasmin. The homoserine content of Facb C-3 was found to be 0.1% after deduction of the homoserine contributed by the C-4A and C-4B contaminants (see below).

Table 1. *Amino acid analysis of CNBr peptide C-3 prepared from Facb and from IgG*

Values for IgG C-3 and Facb C-3 were calculated from Mole (1971).

	Amino acid (mol/mol of peptide)		
	IgG C-3	Facb C-3	Facb C-3 found
Lys	7	6	5.8
His	2	2	1.6
Arg	7	6	5.6
Asp	7	7	8.2
Thr	8	7	6.6
Ser	5	5	4.8
Glu	15	13	12.3
Pro	9	7	7.3
Gly	2	1	2.3
Ala	5	4	4.4
Val	11	10	9.8
Ile	6	6	5.3
Leu	5	4	4.8
Tyr	2	1	1.3
Phe	3	3	3.0
Hser	1	—	<0.1*
Trp	2	2	not determined
Cys	2	2	1.7†

* Determined separately from peptide T14.

† Calculated from ^{14}C labelling of *S*-carboxymethylcysteine.

Table 2. *Carboxypeptidase B digestion of Facb C-3*

Time (h)	Amino acid released	(mol. %)
0	—	0
0.25	Lysine	46
0.5	Lysine	52
1	Lysine	56
16	Lysine	89

Reduction and alkylation of Facb C-3

Facb C-3 was dissolved at a concentration of 10mg/ml in 7M-guanidine hydrochloride, 0.5M-Tris–

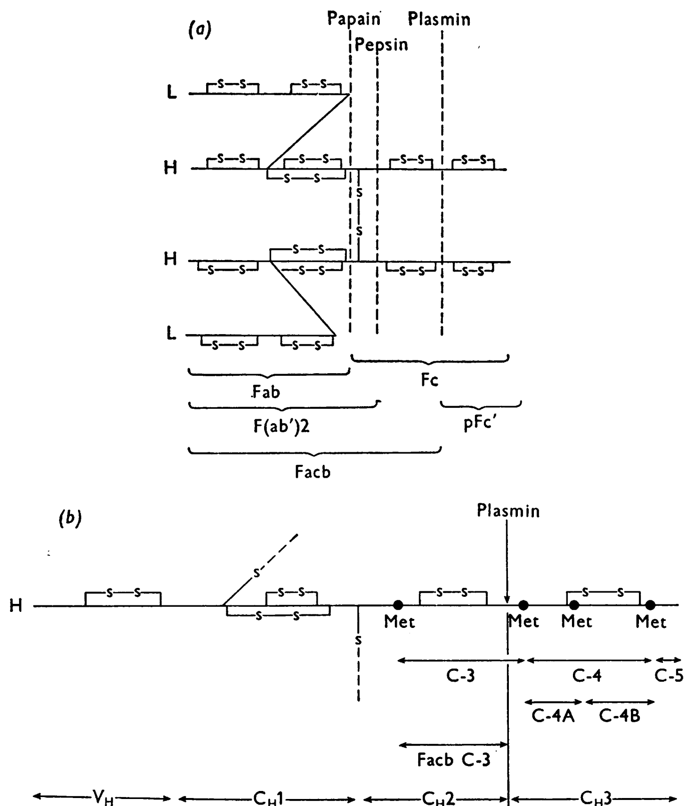


Fig. 1. *Structure of IgG*

(*a*) Proteolytic fragments of rabbit IgG. Papain hydrolysis cleaves IgG into two Fab fragments and one Fc fragment. Pepsin cleavage gives one $F(ab')_2$ fragment including the inter-heavy chain disulphide link and several peptides representing splits in the Fc region, among which was pFc′. (*b*) Position of the split by plasmin in rabbit IgG heavy chain. Methionine residues and CNBr fragments in the Fc portion of the chain are shown. The homology regions are V_H1, C_H1, C_H2 and C_H3.

HCl, pH8.0, 2mM-EDTA and reduced with 50mM-dithiothreitol for 3h at 37°C. Iodo[^{14}C]acetic acid was added in 1.5 molar excess over the thiol groups and after further incubation for 15min at 37°C the total sample was applied to a column of Sephadex G-100 equilibrated with 6M-urea–0.2M-sodium formate, pH3.3. The elution profile (Fig. 2) shows a major peak corresponding to Facb C-3 (recovery about 80%) and two minor peaks corresponding to contaminants C-4B and C-4A.

The fraction containing Facb C-3 was dialysed at 4°C against 0.2M-acetic acid. Some 96% of the ^{14}C radioactivity was recovered in the diffusate and the alkylated Facb C-3 fraction was freeze-dried.

Tryptic peptides from Facb C-3

^{14}C-labelled Facb C-3 and IgG C-3 fractions were digested with trypsin and the peptides isolated.

The presence of 14 peptides (Hill *et al.*, 1967) in fraction C-3 prepared from IgG was verified by separation and analysis by paper electrophoresis. They correspond to the sequence shown in Fig. 3.

By direct comparison three peptides were clearly missing in the tryptic digest of Facb C-3, including one arginine-positive peptide. These peptides from IgG C-3 were separated by one-dimensional electrophoresis and analysed. The percentage yield obtained by parallel elution of IgG C-3 and Facb C-3 tryptic peptides from the electrophoretograms are given in Table 3. They point to a major split by plasmin between peptides 11 and 12.

Determination of ^{14}C radioactivity in peptides T2 and T8 shows a nearly equal distribution between the two carboxymethylcysteines (Table 4). This was also checked by radioautography on the electrophoretograms, which after prolonged exposure showed only

two major radioactive spots of apparently equal intensity. With the high recovery of radioactivity in the reduced Facb C-3 peptide and the detection of only *N*-terminal isoleucine, this is good evidence against any significant hydrolysis occurring within the C-3 sequence.

Fig. 2. *Chromatography of reduced and alkylated Facb C-3 on Sephadex G-100 in 6M-urea–0.2M-sodium formate, pH3.3*

Facb C-3 (10mg) was loaded on to the column (80cm × 3cm) of Sephadex G-100. ●, E_{280}; ○, ^{14}C radioactivity.

Complement binding

As shown in Fig. 4 complement binding by Facb is of the order of 55–60% of the original IgG and 85–90% of the corresponding control acid-treated

Table 3. *Yield of peptides T11, T12, T13 and T14 from IgG C-3 and Facb C-3*

The amino acid sequences and positions of the peptides are shown in Fig. 3. Mobilities are given relative to ε-Dnp-lysine = 0, lysine = +1.0. Percentage recovery was calculated by taking the recovery in parallel of the corresponding peptides from a C-3 tryptic digest as 100%.

Peptide no.	Electrophoresis pH	Electrophoresis Mobility	% of recovery
T11	1.9	+0.60	75.0
T12	3.5	+0.81	7.8
T13	1.9	+0.38	8.4
T14	1.9	+0.07	6.3

Table 4. *Distribution of ^{14}C radioactivity in peptides T2 and T8 from Facb C-3 and IgG C-3*

Radioactivity was determined on the eluted peptides. Recovery from paper was 76.5% of that applied for IgG C-3 and 68.0% for Facb C-3.

Peptide no.	^{14}C radioactivity (d.p.m.)		^{14}C radioactivity (% of total)	
	IgG C-3	Facb C-3	IgG C-3	Facb C-3
T2	2720	3625	55	57
T8	2045	2950	45	43

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     ←—T1—→ ←——————————————T2————————————
(25) Ile-Ser-Arg-Thr-Pro-Glu-Val-Thr-Cys-Val-Val-Asp-Val-
     ——————————————CHO?———————————————————————————
     Ser-Glx-Asx-(Asx,Pro,Glx,Val,Glx)Phe-Thr-Trp-Tyr-Ile-Asp-
     ——————————————→ ←——————T3——————————→←————
     Asx-Glx-Gln-Val-Arg-Thr-Ala-Arg-Pro-Pro-Leu-Arg-Glu-Gln-
     ————————CHO— T4 ——————→←——————— T5 ——————
     Gln-Phe-Asp-Ser-Thr-Ile-Arg-Val-Val-Ser-Thr-Leu-Pro-Ile-Ala-
     ——————————————————→ ←——→ ←—T7—→←———→←————
     His-Glu-Asp-Trp-Leu-Arg-Gly-Lys-Glu-Phe-Lys-Cys-Lys-Val-His-
     ———→ ←———————————————————→ ←—T11———→
     Asp-Lys-Ala-Leu-Pro-Ala-Pro-Ile-Glu-Lys-Thr-Ile-Ser-Lys-
     ←T12→←——————————T13——————————→←——T14———→ (110)
     Ala-Arg-Gly-Gln-Pro-Leu-Glu-Pro-Lys-Val-Tyr-Thr-Met-
                                                  (123)
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Fig. 3. *Tryptic peptides from CNBr fragments C-3*

The residues are numbered from the inter-heavy-chain disulphide bond. Peptides T12, T13 and T14, which are included in the hatched area are missing in Facb C-3.

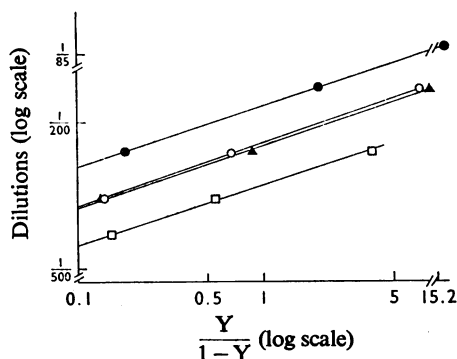

Fig. 4. *Complement binding by Facb*

Complement activity expressed as CH_{50} was calculated as described by Mayer (1961). 100Y is the percentage of haemolysis. The amount of antibody protein incubated with undiluted guinea-pig serum was 94 μg for IgG (●), 95 μg for IgG incubated at pH 2.5 but not treated with plasmin (○) and 97 μg for Facb (▲). Untreated guinea-pig serum (□) had an activity of 294 CH_{50} units/ml.

Table 5. *Relative complement binding by the fractions eluted from Sephadex G-150*

The values were calculated on a molar basis, taking mol.wt. of IgG as 150000 and mol.wt. of Facb as 130000. Digestion by plasmin was 10 min for (*a*) and 18 h for (*b*). Experimental details are given in the text.

	Complement binding		C1 binding
	(*a*)	(*b*)	(*a*)
IgG	100	100	100
IgG, acid-treated	63	62	72
Facb	56	32	69

IgG. This result points to a clear-cut effect of the acid treatment of IgG, which lowers the complement binding by 35–40%. The C1 binding by the Facb–ovalbumin complex (Table 5) was found also to be 85–90% of the binding by the acid-treated IgG–ovalbumin complex. Here again a loss of C1 binding was found of the same order as that for the complement binding described above, on treatment of the IgG at pH 2.5. These results indicate clearly that Facb is able to bind and activate C1.

Discussion

In the preliminary work on the isolation of Facb (Connell & Porter, 1971) from a plasmin digest of rabbit IgG, the major difficulties lay in the separation of Facb from undigested IgG and in prevention of subsequent slow digestion of Facb by the persistent contamination with plasmin. No satisfactory method of fractionation of Facb and IgG could be found, but study of the conditions of pretreatment and digestion led to a method in which the IgG remaining was decreased to about 5%. Decreasing the time of incubation at pH 2.5 and 30°C to less than 15 min resulted in increased amounts of undigested IgG, whereas longer times were of no advantage. Extending the digestion period with plasmin beyond 30 min showed evidence of further digestion and loss of complement-fixing activity (Table 5). The final conditions appear to be optimum, but the content of IgG could not be brought to less than 5%.

Addition of soya bean trypsin inhibitor prevented any further hydrolysis and enabled the Facb to be isolated under mild conditions for complement-fixation studies. For chemical investigation, addition of extraneous protein was undesirable and it was essential to remove any traces of absorbed peptides and hence the more drastic conditions of fractionation in acid urea solutions were chosen. Comparison by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis showed the products obtained by the two methods to be identical chemically.

The presence of only isoleucine and lysine as *N*- and *C*-terminal residues respectively in the C-3 peptide, the high recovery of ^{14}C-labelled *S*-carboxymethylcysteine and its equal division between the two peptides containing *S*-carboxymethylcysteine is strong evidence against any internal splits within the Facb C-3 peptide. The 6–8% recovery of the three *C*-terminal tryptic peptides of Facb C-3 agrees with the estimated 5% contamination of Facb with IgG and the high recovery of the adjacent peptide shows that the major plasmin split occurs at the lysine–alanine bond in the sequence Thr-Ile-Ser-Lys-Ala-Arg. As the recovery of peptides from electrophoresis on paper may vary within at least 10–15%, splitting at lysine bonds nearer the *N*-terminal of Facb cannot be excluded, but, if it occurs, it must be confined to only a small fraction of the molecules. No plasmin hydrolysis occurs in the hinge region of IgG, long known to be vulnerable to many proteases, presumably because the only lysine residue in the sequence of the rabbit heavy chain in this section is followed by proline. In the human IgG1, on the contrary, plasmin can hydrolyse a lysine–threonine bond in the hinge region (Connell & Painter, 1966) as well as causing a split between the C_H2 and C_H3 domains of the Fc fragment after acid treatment (Ellerson *et al.*, 1972). Hence so far, Facb has been prepared only from rabbit IgG.

Accurate measurement of the complement-fixing ability of Facb from an antibody after precipitation with antigen is difficult for several reasons. First, the rate of precipitation of Facb is markedly decreased compared with that of the whole antibody and standing at 2–4°C for 16 h after reaction for 1 h at 37°C is necessary for complete precipitation. Secondly, the pretreatment of IgG containing anti-ovalbumin at

pH2.5 leads to aggregation of a part of the IgG on bringing back to neutrality. Unless the aggregate is removed by fractionation on a Sephadex column this leads to increased precipitation on subsequent addition of ovalbumin. The increase may be as much as 50–100%, but it is specific in the sense that addition of an unrelated antibody, rabbit anti-(bovine serum albumin), to acid-treated anti-ovalbumin followed by precipitation with serum albumin does not bring down with it any aggregated protein. The conformational change of the Fc region caused by acid is only partially reversed at neutrality and leads to interaction between the Fc section of the antibody and non-antibody IgG and hence increased precipitation. This effect appears to be confined to the C_H3 domain, as little if any co-precipitation occurs with Facb. This phenomenon may be of importance when working with antibody purified by affinity chromatography on antigen–Sepharose columns if antibody is recovered by dissociation in acid, as is used frequently.

After removal of aggregate, the weights of antibody precipitated by antigen from IgG, before and after acid treatment, and from Facb are similar. The units of complement fixed by preformed antibody–antigen precipitates of Facb and acid-treated IgG are close, though both are significantly less than those fixed by native antibody. When the first component of complement is determined the same result is obtained, i.e. Facb fixes complement by the classical pathway via C1 as efficiently as whole IgG subjected to the same acid treatment. As rabbit $(Fab')_2$ does not bind C1 (Reid, 1971) the whole binding site for C1 is likely to be in the C_H2 domain, but some irreversible damage is caused by acid. Determination of carbohydrate in IgG and Facb showed no significant difference (M. Colomb, unpublished work). The nature of the secondary effect of the acid remains unidentified.

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