Enzymic Degradation of the Fc Fragment of Rabbit Immunoglobulin IgG

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The digestion of the Fc fragment of rabbit immunoglobulin IgG by several proteolytic enzymes was investigated by using gel filtration and starch-gel electrophoresis in 8M-urea–formate as criteria of the extent of degradation. Though fragment Fc and mildly reduced fragment Fc proved resistant to tryptic hydrolysis, papain and pepsin cleaved the fragment at acidic pH values and appeared to give rise to a similar spectrum of products. A (limit) peptide comprising the *C*-terminal 113 residues of the heavy chain was isolated and identified from the pepsin-digest products of fragment Fc. The products of proteolytic digestion of fragment Fc were no longer able to inhibit passive cutaneous anaphylaxis by rabbit anti-(bovine serum albumin) or demonstrate reversed passive cutaneous anaphylaxis in the guinea pig. Nor were they able to inhibit the intestinal absorption of heterologous immunoglobulin IgG in the young mouse. These studies imply that the site or sites responsible for these biological properties of intact fragment Fc reside in the *N*-terminal 30–40% of the fragment.

Papain digestion of rabbit immunoglobulin IgG has been demonstrated to cleave the molecule into two functionally distinct types of fragments, i.e. fragment Fab, containing the antibody-combining site, and fragment Fc (Porter, 1959) (the nomenclature used is that recommended by the World Health Organisation, 1964). The Fc fragment appears to possess the site or sites responsible for such diverse biological properties of the original molecule as skin sensitization (Ovary & Karush, 1961), placental transfer (Brambell, Hemmings, Oakley & Porter, 1960), intestinal absorption (Morris, 1963) and control of catabolism (Spiegelberg & Weigle, 1965). Conversely, pepsin digestion of rabbit immunoglobulin IgG at pH 4·5–5, which is known to extensively degrade the Fc part of the molecule (Nisonoff, Wissler, Lipman & Woernley, 1960), has been shown to be associated with a loss of complement-fixing activity (Taranta & Franklin, 1961) and cytophilicity (Berken & Benacerraf, 1966), implying that the sites for these biological properties also reside in this area.

The present attempt to hydrolyse fragment Fc, with retention of biological activity, was undertaken in an effort to relate biological function to structural features of the molecule. Though this goal was not achieved, recent advances in our knowledge of the primary structure of rabbit fragment Fc (Givol & Porter, 1965; Hill, Delaney, Lebovitz & Fellows, 1966) made it possible to position a large-digest fragment with certainty. Conclusions on the further degradation of fragment Fc have been drawn.

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MATERIALS

Rabbit immunoglobulin IgG. This was prepared from serum, obtained from a rabbit farm, by precipitation with Na_2SO_4 (Kekwick, 1940) followed by chromatography on DEAE-cellulose in 25mM-potassium phosphate buffer, pH 6·9. The unretarded protein contained only immunoglobulin IgG, as judged by electrophoresis on cellulose acetate strips or by immunoelectrophoresis.

Fragment Fc. This was prepared from a papain digest of rabbit immunoglobulin IgG (Porter, 1959). After 18hr. the digestion mixture was dialysed against 5mM-potassium phosphate buffer, pH 7·4, at 4°. The resultant precipitate was suspended in 0·1M-tris–HCl buffer, pH 8·2, and 5N-NH_3 was added dropwise until the precipitate dissolved, usually at pH 9·6. The solution was centrifuged at 14000*g* for 30min., dialysed in the cold against 0·1M-tris–HCl buffer, pH 8·2, and the crystalline precipitate was recrystallized twice according to the above procedure.

Fragment Pep-III'. This was prepared from the peptic digest of immunoglobulin IgG carried out by the method described by Nisonoff *et al.* (1960), except that ammonium acetate buffer was used in place of sodium acetate buffer; the reaction mixture was made 1mM with respect to *p*-chloromercuribenzoate to minimize disulphide interchange. Isolation of the fragments was achieved by gel filtration on Sephadex G-75 in 50mM-NH_4HCO_3 (Utsumi & Karush, 1965).

Enzymes. Papain, carboxypeptidase A, chymotrypsin and trypsin were purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Chymotryptic activity of the trypsin was minimized by treatment with chloromethyl L-2-phenyl-1-toluene-*p*-sulphonamidoethyl ketone as described by Kostka & Carpenter (1964). Carboxypeptidase A was treated with di-isopropyl phosphorofluoridate, as described by Potts, Young, Anfinsen & Sandoval (1964). Pepsin purified by chromatography (Ryle & Porter, 1959) was a gift from Dr A. P. Ryle.

Antisera. Antiserum specific for rabbit Fc fragment, prepared in a goat, contained approx. 2mg. of precipitable antibody/ml. (Fleischman, Pain & Porter, 1962). Anti-(bovine serum albumin) and anti-(bovine immunoglobulin IgG) sera were prepared in rabbits as described by Porter (1955). A precipitating antiserum against bovine immunoglobulin IgG was produced in C_{57} mice (Morris, 1964); to minimize the appearance of spurious cross-reactions in the intestinal-absorption studies, this antiserum was absorbed with the serum of 9–12-day-old mice.

Anhydrous hydrazine. This was prepared from hydrazine hydrate (Kusama, 1957).

METHODS

The following methods were used, as described by Press, Piggot & Porter (1966): *N*-terminal amino acid determination by fluorodinitrobenzene; thin-layer chromatography for phenylthiohydantoin derivatives; cyanogen bromide cleavage; paper electrophoresis and starch-gel electrophoresis in urea; specific stains; amino acid analysis. Methionine and half-cystine were determined after performic acid oxidation (Moore, 1963). Subtractive Edman degradation was performed by the method described by Konigsberg & Hill (1962) and cyclization was accomplished at pH1·0 in aq. 30% (v/v) ethanol–HCl (Ilse & Edman, 1963). The Bradbury (1958) modification of hydrazinolysis was employed to determine *C*-terminal residues. Peptide mobilities on paper electrophoresis are reported relative to lysine markers.

Reversed passive cutaneous anaphylaxis and inhibition of direct passive cutaneous anaphylaxis were carried out as described by Ovary (1964). In reversed anaphylaxis, 0·1ml. of each fraction (6–400μg./ml.) was injected intradermally and 3hr. later 0·5ml. of 1% Evans Blue dye and 0·1ml. of goat anti-(rabbit Fc fragment) serum were given in a foot vein. To test the digest products for capacity to inhibit direct anaphylaxis by rabbit anti-(bovine serum albumin), the antiserum was diluted to 2·5μg. of antibody/ml. with solutions of the digest (0·4–8·0mg./ml.); 0·1ml. of each mixture was injected intradermally, and 3hr. later 0·5ml. of dye and 0·1ml. of serum albumin were injected intravenously. The largest diameter of the blue area of the skin was read after 30min.; assays were done in triplicate.

Inhibition of intestinal absorption of bovine immunoglobulin IgG in 9–12-day-old mice was carried out as described by Morris (1964). Solutions for feeding to mice were made up such that the final concentration was 1% (w/v) with respect to the bovine immunoglobulin IgG. The concentration of material to be studied for inhibition was varied from 0·04 to 0·5% (w/v). At 4hr. after the gastric feeding the animals were killed and bled by cardiac puncture. Bovine immunoglobulin IgG in the young mouse sera was estimated by the Preer modification of the Oakley–Fulthorpe method as described by Morris (1964).

RESULTS

Enzyme digestion of fragment Fc

Trypsin. Recrystallized fragment Fc proved to be extremely resistant to tryptic degradation. When followed in the pH-stat, 6hr. incubation (37°) at pH8·2 with 2% (w/w) trypsin cleaved a maximum of 5·5 bonds/Fc fragment of mol.wt. 50000, assuming pK 7·3 for the α-amino group (Murachi & Neurath, 1960). On gel filtration through Sephadex G-100 or on starch-gel electrophoresis in 8M-urea–formate the trypsin-treated fragment Fc behaved identically with the control fragment Fc. Mild reduction in 0·1M-mercaptoethanol followed by alkylation with iodoacetamide (Fleischman *et al.* 1962) did not appear to alter the susceptibility of the Fc fragment to trypsin digestion, since under the above conditions of digestion a

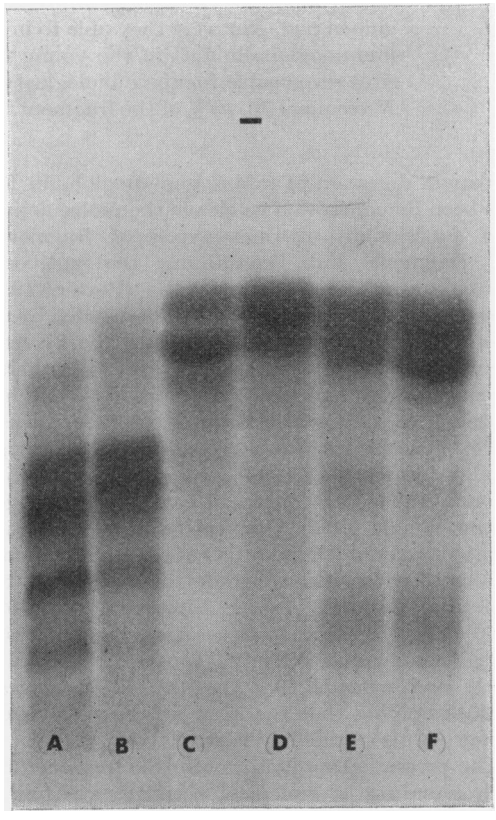

Fig. 1. Starch-gel electrophoresis in 8M-urea–formate buffer, pH3·7, of fragment Fc and digest products. (A) Control fragment Fc; (B) mildly reduced fragment Fc; (C) fragment Pep III′ (Fc); (D) fragment Pep III′; (E) papain digest of fragment Fc at 2hr.; (F) papain digest of fragment Fc at 7hr.

maximum of 4·7 bonds/Fc fragment of mol.wt. 50000 were broken.

Pepsin. Fragment Fc proved to be susceptible to further digestion by pepsin, as observed by Goodman (1964). After 18hr. incubation at 37° at pH4·5, less than 10% of intact fragment Fc remained, as demonstrated by starch-gel electrophoresis in urea. Fragment Fc, as prepared by the method of Porter (1959), characteristically gives three major bands on starch-gel electrophoresis in 8M-urea–formate (Fig. 1A), the more cationic double bands representing monomeric forms of fragment Fc, the slower single band a dimeric form. Often traces of a still slower cationic component are observed, and these are considered to represent the 5·1S papain intermediate described by Nelson (1964). Prior reduction and alkylation of fragment Fc leads to diminution or disappearance of the dimeric component (Fig. 1B). After pepsin digestion the characteristic pattern of fragment Fc was replaced by a more cationic double band. The component responsible for the double band (Fig. 1C), which will be called fragment Pep III′ (Fc), was isolated from the pepsin digest of fragment Fc by gel filtration on Sephadex G-75 in 50mM-ammonium hydrogen carbonate, where it was eluted in a position equivalent to 52–56% of the total column volume, as was fragment Pep III′. Indeed, the elution pattern of the peptic digest of fragment Fc was similar to that of the peptic digest of whole immunoglobulin IgG (Utsumi & Karush, 1965) except that the excluded material (fragment Fab′$_2$) was absent in the former case. Fragment Pep III′ also gave a double band on starch-gel electrophoresis in 8M-urea–formate (Fig. 1D).

Papain. Below pH5·0 the digestion of fragment Fc by papain was found to be extensive when followed by a combination of starch-gel electrophoresis in 8M-urea–formate and gel filtration on Sephadex G-75. Variation of the digestion pH in the range 3·1–4·7 affected the degree of degradation of fragment Fc and multiplicity of banding on starch-gel electrophoresis in urea. Between pH3·4 and 3·7 two new predominant bands were formed (Figs. 1E and 1F), reminiscent of the pattern given by fragment Pep III′ (Fc) or fragment Pep III′. The component in the papain digest responsible for the double banding was isolated by gel filtration on Sephadex G-75 in 50mM-ammonium hydrogen carbonate, where it was eluted with the same relative column volume as was fragment Pep III′ (Fc); in fact, the gel-filtration profile of the digest

Table 1. *Amino acid compositions of fragments of fragment Fc, and* N- *and* C-*peptides of fragment Pep III′*

	Fc*	Pep III′ of Fc*	Residues 1–113 of Fc†	Papain fragment of Fc*	*N*-peptide of Pep III′	*C*-peptide of Pep III′
Ala	7·9	4·2	4	4·3	1·00	1·00
Arg	10·9	5·1	5	4·8	0·83	0·92
Asp	19·2	9·9	9	10·2	0·07	0·92
Cys‡	4·6	2·2	2	2·3	—	—
Glu	23·6	10·8	11	11·3	1·73	1·91
Gly	10·7	7·0	7	7·3	1·29	1·16
His	4·9	3·1	3	3·2	0·03	2·67
Ile	9·7	3·7	4	3·8	0·89	0·89
Leu	14·6	7·3	7	7·8	0·95	0·92
Lys	13·5	7·7	8	7·3	3·03	0·99
Met‡	3·7	2·7	3	2·9	1·02‖	—
Phe	7·7	3·2	3	3·3	—	—
Pro	20·8	7·6	8	7·9	1·87	1·20
Ser§	19·0	13·4	16	14·8	0·89	2·65
Thr§	15·4	7·1	8	7·8	1·61	0·92
Trp	N.D.	N.D.	2	N.D.	—	—
Tyr	7·4	4·7	6	7·4	0·77	0·75
Val	16·7	7·7	7	7·9	0·95	—
Total residues			113		18	18

N.D., Not determined.

* Reported as residues/monomer; molecular weights of monomers were: fragment Fc, 25000; fragment Pep III′ (Fc) and papain fragment of fragment Fc, 13500.

† Derived from the sequence of Hill *et al.* (1966).

‡ Analysed as cysteic acid and methionine sulphone after performic acid oxidation (Moore, 1963).

§ Uncorrected for destruction.

‖ Analysed as homoserine.

was strikingly similar to that given by the peptic digest of fragment Fc. The yield of the component responsible for the two bands was sensitive to variation in digestion time, pH and temperature, whereas digestion of fragment Fc to small peptides could be accomplished by prolonged digestion at any pH. A maximal yield of 1·0–1·2moles of the papain fragment/50000g. of fragment Fc (50–60% of theoretical based on amino acid compositions; Table 1) was achieved by gel filtration of the digest of a 1% (w/v) solution of Fc that had been incubated at 37° in glycine–hydrochloric acid buffer, pH 3·4 and I 0·1, for 7hr. at a papain concentration of 0·01% (w/v). In these studies the papain was always activated before being introduced into the digestion mixture, although the incorporation of reducing reagent did not appear to alter the course of digestion significantly. It was observed, however, that in the absence of additional thiol or chelating agents acetate buffer interfered with digestion, presumably owing to the presence of thiol poisons. The use of formate or glycine–hydrochloric acid buffers overcame this difficulty.

Comparison of fragment Pep III′, fragment Pep III′ (Fc) and the papain fragment of fragment Fc

As described, the three fragments, i.e. fragment Pep III′, fragment Pep III′ (Fc) and the papain fragment of fragment Fc, were isolated by gel filtration of the respective digests on Sephadex G-75, where they were eluted in identical column volumes. On immunodiffusion against goat anti-(rabbit fragment Fc), fragment Pep III′ (Fc) gave a reaction of identity with fragment Pep III′. Similarly the papain fragment of fragment Fc also gave a reaction of identity with fragment Pep III′ (Fc) (Fig. 2), and both appeared to lack a determinant or determinants of fragment Fc, as observed by Utsumi & Karush (1965) for fragment Pep III′. The amino acid composition of fragment Pep III′ (Fc), given in Table 1, was identical with that obtained on analysis of my preparation of fragment Pep III′. The marked similarity in amino acid composition of the papain fragment of fragment Fc to that of fragment Pep III′ (Fc) is also shown in Table 1. Hydrazinolysis revealed glycine to be the C-terminal residue of fragment Pep III′ (Fc) (1·6moles/27000g., uncorrected). Glycine was also established as the major C-terminal residue of the papain fragment by hydrazinolysis, but the yield of glycine was poor (0·8mole/27000g., uncorrected). Bis-DNP-lysine (1·4moles/27000g., uncorrected), DNP-alanine (0·1 mole/27000g.) and DNP-serine (0·1mole/27000g.) were identified as N-terminal residues of fragment Pep III′ (Fc). The same results were obtained when Pep III′ was subjected to C- and N-terminal analysis. Thus by the criteria of gel filtration, starch-gel electrophoresis in urea, immunodiffusion, amino acid composition and N- and C-terminal analysis the fragment Pep III′ (Fc), isolated from the peptic digest of fragment Fc, was identical with fragment Pep III′ prepared according to the procedure described by Utsumi & Karush (1965). Although the papain fragment of fragment Fc also bore marked similarities to fragment Pep III′, complete characterization of the fragment was not undertaken. It must be pointed out that the papain fragment of fragment Fc described here contained insignificant amounts of carbohydrate (less than 0·1mole/27000g.) as judged by hexosamine content (Cessi, 1952), in contrast with fragment Pap III′, a fragment that Utsumi & Karush (1965) isolated as a papain degradation product of fragment Fc.

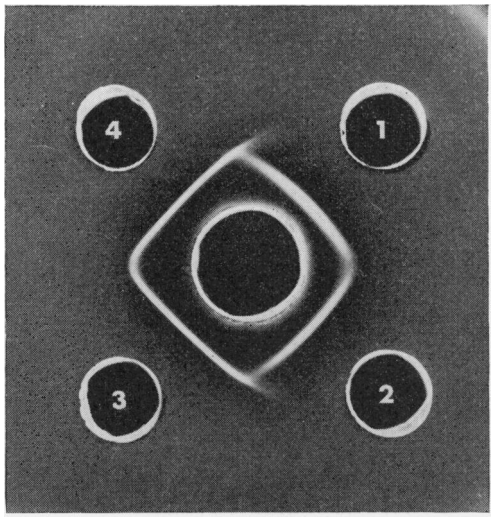

Fig. 2. Immunodiffusion of fragment Fc and fragments of fragment Fc against goat anti-(fragment Fc). Well no. 1, fragment Pep III′ (Fc) at 0·41mg./ml.; well no. 2, papain fragment of fragment Fc at 0·45mg./ml.; wells no. 3 and no. 4, fragment Fc at 0·40mg./ml. Centre well, goat anti-(fragment Fc). The pattern was photographed after 48hr. at 25°.

Position of fragment Pep III′ in fragment Fc

Givol & Porter (1965) have reported the isolation and sequence of the C-terminal octadecapeptide from the cyanogen bromide cleavage products of the heavy chain of rabbit immunoglobulin IgG. It is striking that this peptide contains three histidine residues/mol., whereas only five such residues are found in the whole Fc section of the heavy chain (Table 1). The presence of three

histidine residues/fragment Pep III′ of mol.wt. 13500 and the same *C*-terminal residue (glycine) suggested that fragment Pep III′ might be derived from the *C*-terminal end of the heavy chain (Silman, Cebra & Givol, 1962). Therefore the investigation of the products of cyanogen bromide cleavage of fragment Pep III′ was undertaken. A fraction absorbing at 280mμ was eluted at 76% of the total column volume on gel filtration of the reaction mixture on Sephadex G-50 in N-acetic acid. The fraction was pooled and freeze-dried. On paper electrophoresis at pH 3·5 this fraction moved as a broad spreading band with average mobility 0·75–0·82 (relative to lysine) and gave positive ninhydrin, histidine and arginine staining reactions, but a negative tryptophan staining reaction. Two components (*N*- and *C*-peptides) were resolved by paper electrophoresis at pH 6·5 (mobilities 0·61–0·64 and 0·38–0·41 respectively), or more cleanly in 20mM-*N*-ethylmorpholine–acetate buffer, pH 8·0 (mobilities 0·45 and 0·21 respectively). Only the slower component (*C*-peptide) stained for histidine, and neither could be resolved further either by electrophoresis at pH 1·9 or by paper chromatography in butan-1-ol–acetic acid–water (3:1:1, by vol.) or pyridine–3-methylbutan-1-ol–water (7:7:6, by vol.). Analysis of the *N*- and *C*-peptides is given in Table 1. The amino acid composition of the original fraction eluted from Sephadex G-50 could be accounted for by summation of these two components. Recoveries of the *N*- and *C*-peptides were 70% and 60% respectively, assuming three methionine residues/mol. of fragment Pep III′ monomer (mol.wt. 13500; Utsumi & Karush, 1965).

C-Peptide. The *C*-peptide contained no homoserine. Glycine was confirmed as the *C*-terminal residue by hydrazinolysis (0·6mole/mole, uncorrected), establishing the peptide as *C*-terminal in fragment Pep III′. A single step of the Edman degradation produced a decrease in the histidine content from 2·67 to 1·89moles/mole, fixing this residue in the *N*-terminal position. The amino acid composition of the *C*-peptide was the same as that reported by Givol & Porter (1965) for the *C*-terminal octadecapeptide of rabbit immunoglobulin IgG heavy chain. Digestion of *C*-peptide with trypsin (0·01%) at 25°, for 2hr. yielded three peptides on paper electrophoresis at pH 6·5 of mobilities 0·65, 0·57 and 0·0; the staining characteristics and compositions of these peptides were identical with those described for the tryptic peptides of the heavy-chain octadecapeptide (Givol & Porter, 1965). Since the data of Hill *et al.* (1966) ruled out the possible repetition of this sequence in the Fc part, fragment Pep III′ must represent a *C*-terminal fragment of the heavy chain.

N-Peptide. The *N*-terminal residue of the *N*-peptide was identified as lysine (0·7mole of bis-DNP-lysine/mole, uncorrected), suggesting that this peptide was derived from the *N*-terminal part of fragment Pep III′. Since examination of a cyanogen bromide digest of fragment Pep III′ that had been treated with fluorodinitrobenzene revealed no increase in bis-DNP-lysine over that observed in fragment Pep III′, an *N*-terminal lysine residue was not produced during cyanogen bromide cleavage. The *N*-peptide was not detected in a cyanogen bromide digest of fragment Fc, and must have resulted from the removal of the *N*-terminal portion of fragment Fc by peptic digestion.

The peptides formed from *N*-peptide by chymotryptic digestion (0·01%) at 37° for 2hr. in 0·1M-ammonium hydrogen carbonate were subjected to gel filtration on Sephadex G-25 in 20mN-ammonia; the extinction of the effluent was measured at 215mμ. Three peaks, *NC*1, *NC*2 and *NC*3 (elution volumes 68%, 55% and 38% of the total column volume respectively), were obtained, pooled and concentrated. Three peptides were separated from peak *NC*1 by electrophoresis at pH 6·5, the characteristics and compositions of which are shown in Table 2. Edman degradation and hydrazinolysis established the sequence of peptide *NC*1b as Lys-Thr-Ile-Ser (Table 3). Peptide *NC*1a was shown to be an extension of peptide *NC*1b (Table 4) and to

Table 2. *Amino acid compositions and characteristics of the peptides of peak* NC1

The compositions are given as moles of amino acid/mole of peptide.

	*NC*1a	*NC*1b	*NC*1c
Lys	1·95	1·02	—
Ile	1·00	1·00	—
Ser	1·03	1·07	—
Thr	0·92	0·81	0·89
Ala	1·00	—	—
Homoserine	—	—	1·00
Yield (%)	30	40	80
Mobility at pH 6·5	0·64	0·41	0·0
Ninhydrin colour	Blue	Blue	Yellow
Hydrazinolysis	Ala	Ser	—

Table 3. *Subtractive Edman degradation of* NC1b *peptide*

The amino acid content is given as moles/mole. N.D., Not determined.

Edman step ...	0	1	2	3
Lys	**1·02**	**0·19**	N.D.	N.D.
Ile	1·00	1·00	1·00	**0·29**
Ser	1·07	0·80	0·84	0·87
Thr	0·81	**0·67**	**0·24**	0·05

Table 4. *Subtractive Edman degradation of NC1a peptide*

The amino acid content is given as moles/mole.

Edman step ...	0	1	2
Lys	**1·95**	**1·09**	0·88
Ile	1·00	1·00	1·00
Ser	1·03	0·99	1·14
Thr	0·92	**0·90**	**0·09**
Ala	1·00	0·84	0·78

Table 5. *Amino acid compositions and characteristics of peptide NC2 and the tryptic peptides isolated from peptide NC2*

The compositions are given as moles of amino acid/mole of peptide.

	NC2	*NC2T1*	*NC2T2a*	*NC2T2b*
Lys	1·18	—	—	1·10
Arg	0·97	1·00	—	—
Glu	2·32	—	—	2·10
Pro	2·40	—	—	2·20
Gly	1·20	—	—	0·97
Val	0·93	—	1·00	—
Leu	1·00	—	—	1·00
Tyr	0·76	—	0·74	—
Yield (%)	70			
Mobility at pH 6·5	0·21	0·92	0·0	0·0
Ninhydrin colour	Blue	Blue	Blue	Blue

have the sequence Lys-Thr-Ile-Ser-Lys-Ala. Peptide *NC1c*, Thr-homoserine, must be the *C*-terminus of the *N*-peptide.

A major ninhydrin-staining band of mobility 0·21 was found on subjecting peak *NC2* to paper electrophoresis at pH 6·5. *N*-terminal arginine was identified as α-DNP-arginine by paper electrophoresis of the aqueous phase of the hydrolysates of DNP-*NC2* in N-ammonia; the ether-extractable phase revealed no DNP-amino acids. Tyrosine (0·8 mole/mole) was released from peptide *NC2* by carboxypeptidase A, in agreement with the chymotryptic origin of the peptide. Paper electrophoresis at pH 6·5 of a tryptic digest of peptide *NC2* revealed only free arginine and a ninhydrin-staining neutral band. Electrophoresis at pH 3·5 showed the neutral band to be heterogenous, but purification was best achieved by paper chromatography in butan-1-ol–acetic acid–water–*n*-butyl acetate (19:5:25:1, by vol.) (Richmond & Hartley, 1959). The amino acid compositions of peptide *NC2* and the tryptic peptides of peptide *NC2* are shown in Table 5. A rapidly moving peptide (R_F 0·32), *NC2T2a*, was Val-Tyr. The sequence of peptide *NC2T2b* (R_F 0·08) was established by sequential Edman degradation (Table 6)

Table 6. *Subtractive Edman degradation of peptide NC2T2b*

The amino acid content is given as moles/mole. N.D., Not determined.

Edman step ...	0	1	2	3	4	5
Lys	1·10	N.D.	N.D.	N.D.	N.D.	N.D.
Glu	2·10	**1·95**	**1·17**	1·08	**1·00**	**0·27**
Pro	2·20	2·28	**2·15**	**1·35**	1·25	1·25
Gly	**0·97**	**0·31**	0·15	0·07	0·09	—
Leu	1·00	1·00	1·00	**1·00**	**0·15**	—

to be Gly-Glu-Pro-Leu-Glu-Pro-Lys. The neutrality of peptide *NC2T2b* at pH 6·5 implied the amidation of a glutamic acid residue. After the removal of the glutamic acid residue in the second step of the Edman degradation the resultant peptide was still neutral at pH 6·5, and the residue that was removed was identified as the phenylthiohydantoin derivative of glutamine. Therefore the full sequence of peptide *NC2* is Arg-Gly-Gln-Pro-Leu-Glu-Pro-Lys-Val-Tyr.

The three peptides *NC1a*, *NC1c* and *NC2* account for the whole of the *N*-peptide. Peptide *NC1a* must be the *N*-terminus of the *N*-peptide as it accounts for two of the three lysine residues and the third has been identified in the interior of peptide *NC2*. The full sequence of the *N*-peptide of fragment Pep III′ is therefore that given in Fig. 3.

Biological activity

The capacities of the digests and fragments of fragment Fc that have been described to produce passive cutaneous anaphylaxis in the guinea pig (Ovary, 1964) and inhibit intestinal absorption of heterologous immunoglobulin IgG in the young mouse (Morris, 1964) were compared with those of fragment Fc. Both reversed passive cutaneous anaphylaxis and inhibition of direct passive cutaneous anaphylaxis were assayed to avoid overlooking smaller peptides that might possess the capacity to compete for sensitizing sites but lack anti-(fragment Fc) determinants. In the latter assay 80 μg. of fragment Fc inhibited completely the cutaneous anaphylactic reaction elicited by 0·25 μg. of rabbit anti-(bovine serum albumin); the antiserum alone gave blue lesions averaging 15–17 mm. in diameter. Fragment Fc subjected to tryptic digestion appeared to be as active as undigested fragment Fc. The unfractionated peptic or papain digests showed markedly diminished ability to inhibit the fixation of rabbit anti-(bovine serum albumin) (less than 10% of that given by fragment Fc). When the digests were fractionated by gel filtration on Sephadex G-75 to

Lys-Thr-Ile-Ser-Lys-Ala-Arg-Gly-Gln-Pro-Leu-Glu-Pro-Lys-Val-Tyr-Thr-Met
← *N*C1a → ← *N*C2 → ←*N*C1c→

Fig. 3. Full sequence of the *N*-peptide of fragment Pep III'.

remove undigested fragment Fc (by the criterion of starch-gel electrophoresis in 8M-urea) neither the purified components nor reconstituted mixtures of these showed any activity, even when tested at a tenfold higher molar concentration. Cyanogen bromide-cleaved fragment Fc could not be adequately assayed because of difficulties with insolubility of the products at neutral pH.

Reversed passive cutaneous anaphylactic assays confirmed the above observations; 0·6μg. of fragment Fc produced blue lesions averaging 12–17mm. in diameter, decreasing to 8–13mm. with 0·2μg. of fragment Fc. Once again unfractionated peptic and papain digests of fragment Fc gave weakly positive reactions that could be eliminated by the removal of undigested fragment Fc by gel filtration. Weakly positive reactions were elicited if the large fragments (fragment Pep III' and papain fragment of fragment Fc) were assayed at 20–40μg., but it is unlikely that this represents true sensitization. The smaller peptide material was inactive by this technique, as might have been anticipated.

The assays of inhibition of intestinal absorption of bovine immunoglobulin IgG in the young mouse were carried out on the same characterized digests and fragments. Fragment Fc, reduced fragment Fc and tryptic digests of both gave similar degrees of inhibition. The activity of the unfractionated pepsin digest was markedly less than that due to a one-twelfth molar equivalent of intact fragment Fc, whereas the papain digest showed no inhibiting activity. Fragment Pep III' and the papain fragment of fragment Fc themselves did not inhibit. An uncertainty of the assay, however, resides in the difficulty of maintaining solubility of the components with pH variations occurring after gastric feeding, so that a negative assay might be misleading. There is no evidence that such complications were operative in these studies. It appears therefore that the extensive degradation of fragment Fc incurred in this study was accompanied by total loss of the biological activities investigated.

DISCUSSION

It was not surprising to find that fragment Fc, itself a product of papain cleavage of immunoglobulin IgG, was resistant to further digestion at neutral pH by trypsin or papain. In more acid conditions papain hydrolysis does, however, occur. This pH effect may be due to conformational changes in the substrate such as have been shown to occur in the susceptibility of bovine serum albumin to pepsin (Weber & Young, 1964). It may also be due to change of specificity of papain at acid pH as suggested by the studies of Kimmel & Smith (1954) with peptide substrates. Lastly, it may reflect the state of aggregation of the substrate, as rabbit fragment Fc has been shown to undergo dissociation from dimeric to monomeric units in acid medium (Inman & Nisonoff, 1965).

Utsumi & Karush (1965) reported the formation of a large (limit) fragment, Pep III', from the Fc part of the heavy chain when rabbit immunoglobulin IgG was digested with pepsin according to the method of Nisonoff *et al.* (1960). The present work has shown the formation of an identical (limit) fragment when pepsin was used to digest fragment Fc under the same conditions. Evidence is presented indicating that the same type of fragment is formed by the papain degradation of fragment Fc at low pH, in which case it is not a limit fragment but can be further degraded by the enzyme. On the basis of the antigenic properties of the peptic fragments Utsumi & Karush (1965) proposed a relative positioning of fragment Pep III' in the interior of fragment Fc. The isolation and identification of the same *C*-terminal octadecapeptide from the cyanogen bromide cleavage products of fragment Pep III' as has been reported for the heavy chain (cf. Givol & Porter, 1965) has unequivocably established that fragment Pep III' occupies the *C*-terminal position of the heavy chain of immunoglobulin IgG. Further corroboration was achieved on examination of the primary structure of fragment Fc reported by Hill *et al.* (1966); on counting back to the third methionine residue from the *C*-terminal end (fragment Pep III' contained 2·7 methionine residues/mol. of monomer) the peptide, residues 113–96, which was established as the *N*-terminal peptide of fragment Pep III', was identified. The sequence now reported is the same as that given by Hill *et al.* (1966), except for the placement of a glutamine rather than a glutamic acid residue in position 105. A molecular weight of 25335 for fragment Pep III' dimer, calculated from the sequence given by Hill *et al.* (1966), is in agreement with that of 27000 reported by Utsumi & Karush (1965) based on sedimentation equilibrium. The latter workers observed that fragment Pep III' could be dissociated into its monomeric sub-units without any reductive process, implying that the disulphide bond formed by residues 80 and 22 must be intrachain and not

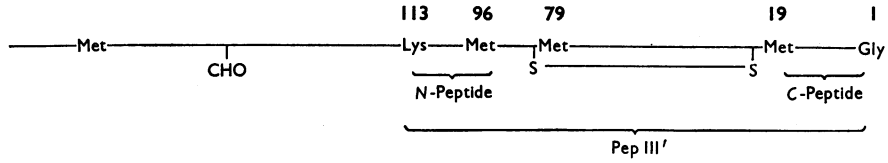

Fig. 4. Fc part of the heavy chain illustrating the relative positions of Pep III′ and the cyanogen bromide cleavage products of fraction Pep III′ discussed in the text. The carbohydrate moiety (CHO) of Fc is also shown.

interchain. A diagrammatic representation of the relative position, in the Fc part of the heavy chain, of fragment Pep III′ and its cyanogen bromide cleavage products is shown in Fig. 4. One minor inconsistency between these data and those of Utsumi & Karush (1965) must be mentioned. Whereas lysine was found to constitute the major *N*-terminal residue of both fragment Pep III′ (Fc) and my preparation of fragment Pep III′, they reported alanine as the major *N*-terminal residue of their preparation of fragment Pep III′. As we both identified the residues of their DNP derivatives, it is possible this difference merely reflects differences in the degree of degradation of the starting material by pepsin. The significance of the double banding given by monomeric fragment Fc and fragment Pep III′ on starch-gel electrophoresis in 8M-urea–formate is not understood.

Unfortunately, the intention to isolate peptides of fragment Fc that still retained biological activity was not realized. Neither the fragments and smaller peptides nor reconstituted mixtures of both demonstrated any capacity to evoke reversed passive cutaneous anaphylaxis or inhibit direct passive cutaneous anaphylaxis in the guinea pig or to inhibit the intestinal absorption of immunoglobulin IgG in the young mouse. Fragment Pep III′ still retains considerable structural integrity as evidenced by its resistance to digestion under the conditions described and its ability to be crystallized (Utsumi & Karush, 1965). Consequently, it appears likely that the site or sites responsible for the biological activities investigated reside predominantly or entirely in that area of fragment Fc extensively degraded by proteolysis, i.e. the *N*-terminal 35–40% of fragment Fc. Goodman (1964) and Paraskevas & Goodman (1965) investigated the intermediate products of partial pepsin digestion of fragment Fc and observed that a general decrease of biological activity was associated with a decrease in size of the components. In conjunction with these data this suggests that the enzymic degradation of Fc occurs in a stepwise fashion from the *N*-terminus and that there is loss of biological activity as the degradation passes through the responsible site or sites on the way to the limit fragment, Pep III′.

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