The Subunit and Polypeptide-Chain Structure of Rabbit Secretory Immunoglobulin A

ISOLATION OF A PROTEOLYTIC FRAGMENT SUITABLE FOR SEQUENCE STUDIES ON THE VARIABLE REGION OF α -CHAIN

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(Received 6 January 1977)

A method was developed for the preparation of a proteolytic fragment of rabbit secretory immunoglobulin A (sIgA) which contains the variable region of the α -chain; this fragment is suitable for primary-sequence studies. The serologically defined subclasses of sIgA are shown to correlate partially with the nature of the binding of a constituent chain of sIgA, called secretory piece. Data are also presented on the relative resistance of sIgA to enzymic and reductive cleavage, compared with immunoglobulin G.

Although rabbit IgG‡ has been well characterized in both its gross and primary structure (Hill *et al.*, 1967; Fleischman *et al.*, 1963; Mole *et al.*, 1971; Pratt & Mole, 1975), less information is available on IgA. IgA is the major class of immunoglobulin in secretions, such as saliva and gastrointestinal fluid, and plays an important part in the body's defence against infection in the immediate environment (for review see Tomasi & Grey, 1972). Also, maternal antibodies of the IgA class are passed to the newborn in milk and protect the offspring until its own immune-response system is fully functional.

Initial structural characterization of sIgA has been reported (Cebra & Small, 1967), and later work (Halpern & Koshland, 1970; O'Daly & Cebra, 1971b) showed that the molecule normally exists as a dimer of the characteristic immunoglobulin subunit (consisting of two heavy and two light chains, mol.wt. 54000 and 22000 respectively) together with two unique polypeptide chains called J-chain (mol.wt. 15000) and secretory piece (mol.wt. 60000). The function of J-chain is covalent linkage of the two subunits (Koshland, 1975), and it is not found in the monomer form of IgA; the function of secretory piece is unknown, although it is found associated exclusively with immunoglobulins in the secretions and not with

serum immunoglobulins (Tomasi & Grey, 1972). Two subclasses of rabbit IgA (encoded by genes at the *f* and *g* loci), each with allotypic variants, have been detected serologically and the antigenic differences between them localized to the α -chain constant region (Hanly *et al.*, 1973).

In the rabbit immunoglobulin system, the serologically defined α -locus allotypic markers, a1, a2 and a3, have been detected in all classes of heavy chain (Oudin, 1960; Todd, 1963; Feinstein, 1963; Kindt & Todd, 1969) and localized to the variable region (Mole *et al.*, 1975). Primary-sequence studies on γ -chains of different allotype showed correlation of the allotype with residues at several positions within the variable region (Wilkinson, 1969a; Mole *et al.*, 1971).

The aim of the studies reported in this and the following paper (Johnstone & Mole, 1977) was to determine the primary sequence of the heavy-chain variable region of rabbit IgA and compare it with that already reported for IgG (Mole *et al.*, 1971). The data obtained would have a 2-fold application. First, they would test the Translocation Hypothesis (Dreyer & Bennett, 1965), which proposes that each immunoglobulin chain is coded for by two genes, one of which, the *V*-gene, is common to all classes of heavy chain (or all types of light chain). Secondly, they would allow re-evaluation of the proposed structural correlates of the α -locus allotypes found in IgG.

The present paper describes the preparation of a fragment (Fd_α), consisting of the *N*-terminal half of α -chain, suitable for use in primary-sequence studies on the variable region, which are reported in the following paper (Johnstone & Mole, 1977). Studies on the gross structure of sIgA are also presented; a

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‡ Abbreviations: IgG, immunoglobulin G; IgA, immunoglobulin A; sIgA, secretory IgA; F(ab')_{2α}, peptic fragment of sIgA analogous to the IgG F(ab')₂ fragment; Fd_α, *N*-terminal half of α -chain analogous to the γ -chain Fd fragment; CmCys, *S*-carboxymethylcysteine; Hse, homoserine; Dnp-, 2,4-dinitrophenyl-.

partial correlation between the subclasses of α -chain and their mode of binding secretory piece is demonstrated.

Experimental

Materials

Papain was purchased from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.), pepsin from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and trypsin from Seravac Laboratories (Pty) Ltd. (Cape Town, S. Africa).

DEAE-cellulose DE-32 was obtained from W. and R. Balston, Maidstone, Kent, U.K. Iodo[$^{14}\text{C}_2$]acetic acid (55 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and stored at -20°C as a 1 mCi/ml solution in water.

Methods

Preparation of sIgA and secretory piece. Rabbits homozygous at the *a*-locus were milked every day from 1 to 3 weeks after parturition. Oxytocin (8 USP units per rabbit) was injected intravenously, milk squeezed from the mammary gland by finger pressure and sucked into vials by means of a water pump. This method yielded 20–30 ml of milk per rabbit per day.

The milk obtained was stored frozen, and sIgA isolated from it as described by Cebra & Robbins (1966) and O'Daly & Cebra (1971a). The only modification was in the DEAE-cellulose chromatography step: the sIgA-containing fraction from 50 ml of milk was applied to a column (2.0 cm $\times$ 30 cm) of DEAE-cellulose in 0.01 M-sodium phosphate (pH 7.5)/0.1 M-NaCl, and sIgA eluted by stepwise application of the same buffer containing 0.25 M-NaCl, at 4°C . The yield of sIgA was 4–5 mg/ml of milk originally taken. This fraction was the starting material for the preparation of the peptic fragment F(ab')_{2a} and for separation of two populations of sIgA.

In some cases, sIgA (150 mg) was further purified and any non-covalently bound secretory piece removed by gel filtration on a column (3.6 cm $\times$ 90 cm) of Sephadex G-200 in 3 M-guanidine hydrochloride at 4°C (Cebra & Small, 1967). The first peak was pure sIgA from which some secretory piece, and possibly some light chain, had been dissociated, and the second peak was pure secretory piece, as judged by polyacrylamide-gel electrophoresis and use of specific antisera.

Cleavage of proteins. CNBr cleavage of polypeptide chains at methionine residues was carried out as described by Steers *et al.* (1965).

Partial reduction and alkylation of interchain disulphide bonds was performed as described by Prah & Porter (1968), and complete reduction and [^{14}C]carboxymethylation of all disulphide bonds was as described by O'Donnell *et al.* (1970).

Peptic digestion of sIgA (600 mg) was carried out in 0.1 M-glycine/HCl, pH 2.4 (protein concentration 10 mg/ml), at an enzyme/substrate ratio of 1:50 (w/w) for 20 h at 37°C (Steward, 1971), the digestion being terminated by neutralization with 2 M-Tris. The fragment F(ab')_{2a} was isolated from the digest by fractionation on a column (5.0 cm $\times$ 90 cm) of Sephadex G-200 in 0.1 M-sodium phosphate, pH 6.8 at 4°C .

Tryptic digestion of sIgA at elevated temperature (60°C) was carried out as described by Zikan *et al.* (1972). The 'IgA-protease' enzyme reported by Plaut *et al.* (1974) was partially purified from the culture medium of *Streptococcus sanguis* and used as described by these authors. Digestion of sIgA by activated papain was performed as described by Hanly *et al.* (1973).

Isolation of fragment Fd_a and the N-terminal 34-residue CNBr fragment of α -chain. The fragment F(ab')_{2a} (200 mg) was partially reduced and alkylated (Prah & Porter, 1968) with 0.1 M-dithiothreitol; excess reagent was removed by dialysis and the protein freeze-dried. The chains were then cleaved with CNBr, the protein was freeze-dried, and then completely reduced and [^{14}C]carboxymethylated, in 7 M-guanidine hydrochloride, by using a 10-fold molar excess of dithiothreitol over cystine residues. The mixture was applied directly to a column (3.0 cm $\times$ 30 cm) of Sephadex G-25 (fine grade) in 0.2 M-sodium formate, pH 3.3, containing 6 M-urea, to remove excess reagents, and the protein pool was left overnight (16 h) at 4°C in the urea buffer to facilitate aggregation of fragment Fd_a. The fragments were then separated by gel filtration on a column (3.2 cm $\times$ 140 cm) of Sephadex G-100 in the same urea buffer.

Antisera. Antisera against the allotypes of the *a*- and *b*-loci were raised by the method of Kelus & Gell (1967). Sheep anti-sIgA was raised by injection of 1 mg of sIgA, emulsified with complete Freund's adjuvant, into the leg muscle. The injection was repeated 4 weeks later and the animal was bled regularly after a further 2 weeks. This antiserum was made specific for IgA (α -chain) by passage down an affinity column consisting of rabbit light chain (from IgG) coupled to Sepharose 4 B by the CNBr method of Cuatrecasas (1970). Elution of the affinity column with 1 M-propionic acid yielded antibodies specific for light chain. Rat anti-(secretory piece) was raised by two intraperitoneal injections of 100 μg of secretory piece, emulsified with complete Freund's adjuvant, at an interval of 1 week.

Analytical methods. Protein fractions were studied serologically by double diffusion and immunoelectrophoresis in 1.5% agar as illustrated by Clausen (1969). The purity of protein fractions was determined as a routine by electrophoresis in 4%, 5.6% or 10% (w/v) polyacrylamide disc gels in sodium dodecyl sulphate (Fairbanks *et al.*, 1971) or in 4% or 5.6% (w/v) polyacrylamide disc gels in alkaline urea [buffer

system of Gordon & Louis (1967) with the gel solution made 6M in urea].

The degree of aggregation of protein fractions was determined by passing 1 mg of each fraction down a column (0.6cm×90cm) of Sepharose 6B in 0.1M-sodium phosphate, pH6.8. From the relative area of the peaks obtained (monitored by A_{280}) an approximate assessment of the amount of protein present in the aggregated and non-aggregated forms was made.

The purity of fragment Fd_α was determined by analysing for the presence of a light-chain marker peptide in the preparation. The C-terminal sequence of b4 κ -chains (which were the major constituents of the light-chain pool from rabbits in our colony) is -Asn-Arg-Gly-Asp-Cys (Frangione, 1969). The cysteine residue, being part of the heavy-light-chain bridge, can be [^{14}C]carboxymethylated after partial reduction of the interchain bonds of fragment $F(ab')_{2\alpha}$ during the preparation of fragment Fd_α (see above). Samples (10nmol) from the pools obtained from the subsequent fractionation procedure were digested with 10 μ g of trypsin in 100 μ l of 1% (w/v) NH_4HCO_3 , pH8.2, and the resultant peptides separated by high-voltage paper electrophoresis at pH3.5. The acidic (charge -2) ^{14}C -labelled tripeptide from light chain was easily identified by radioautography on Kodak BB-54 X-ray film (mobility -0.80 relative to aspartic acid, -1.0, and ϵ -Dnp-lysine, 0).

Amino acid analysis was carried out as described by Pratt & Mole (1975).

Results

Resolution of the sIgA pool into two populations

Two protein bands, of mol.wt. approx. 400000, were observed on polyacrylamide-gel electrophoresis in sodium dodecyl sulphate of sIgA (see Fig. 2, gel *g*); separation of these two proteins was attempted to characterize them structurally. The sIgA fraction (100mg) before treatment with guanidine hydrochloride was applied to a column (2.0cm×30cm) of DEAE-cellulose in 0.01M-sodium phosphate (pH7.5)/0.05M-NaCl, containing 6M-urea, and developed with a linear salt gradient (total volume 600ml) to 0.2M-NaCl in the same urea buffer, all at 4°C. The sIgA eluted was partially separated into two populations (Fig. 1); however, presumably because of variable-region heterogeneity, broad peaks were obtained and complete resolution was not possible.

Pools A and B from this column (Fig. 1) were selected as pure samples of each population, and minor contaminants together with non-covalently bound secretory piece were removed from these pools by gel filtration on a Sephadex G-200 column in 3M-guanidine hydrochloride. This caused the dissociation of some secretory-piece molecules; however, only 5-7% of the A_{280} units recovered from the

Fig. 1. Fractionation of pooled sIgA

Chromatography was conducted at 4°C on a column (2.0cm×30cm) of DEAE-cellulose in 6M-urea/0.01M-sodium phosphate, pH7.5. Arrow indicates commencement of a linear salt gradient (total volume 600ml) from 0.05M- to 0.20M-NaCl. Fraction size was 10ml.

column were present in the secretory-piece fraction. The theoretical secretory-piece content of sIgA is 15.8% by weight (calculated from data presented in the introduction), and so a substantial amount (approx. 50%) of the secretory piece present in sIgA is covalently bound, in agreement with Hanly *et al.* (1973). This conclusion was supported by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate of the sIgA fraction after dissociation with guanidine hydrochloride (Fig. 2, gels *g* and *h*): substantial quantities of secretory piece remained attached to sIgA and could only be separately observed after complete reduction and alkylation.

Secretory piece isolated by gel filtration in guanidine hydrochloride was pure on polyacrylamide-gel electrophoresis, and was free of light chain, as judged by anti-(light chain) sera. The amino acid composition of this protein is shown in Table 1 and *N*-terminal-sequence data have been reported previously (Johnstone & Mole, 1975).

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate of A and B of sIgA separated by DEAE-cellulose chromatography showed that the populations did indeed correlate with the two bands previously observed in sIgA (Fig. 2, compare gels *a*, *c*, *e* and *g*). Electrophoresis of completely reduced and alkylated samples showed that pool B (higher molecular weight) still contained secretory piece, whereas pool A did not (Fig. 2, compare *b*, *d*, *f* and *h*); no

Fig. 2. Polyacrylamide-gel electrophoresis of two populations of sIgA

Electrophoresis was performed in 4% (w/v) polyacrylamide gel in sodium dodecyl sulphate (see under 'Analytical methods'): (a) pool A from Fig. 1; (b) pool A after reduction and alkylation; (c) mixture of pools A and B from Fig. 1; (d) mixture of pools A and B after reduction and alkylation; (e) pool B from Fig. 1; (f) pool B after reduction and alkylation; (g) sIgA; (h) sIgA after reduction and alkylation.

Table 1. Amino acid composition of secretory piece and J-chain

Compositions are given as mol of residues/mol of polypeptide chain, assuming the mol.wt. of the protein portions of secretory piece and J-chain to be 60000 and 13000 respectively (Halpern & Koshland, 1970; O'Daly & Cebra, 1971b). Values for valine and isoleucine were calculated after 72 h hydrolysis and for threonine and serine were corrected for losses on hydrolysis. Cysteine was determined as S-carboxymethylcysteine.

	Secretory piece	J-Chain	
		This study	Koshland (1975)
Lys	29.8	4.4	5.1
His	7.5	1.1	0.9
Arg	21.6	7.7	8.9
Asp	56.7	21.8	22.2
Thr	35.5	12.1	11.7
Ser	42.8	7.1	7.2
Glu	73.4	12.0	11.4
Pro	32.5	9.2	8.7
Gly	47.8	2.7	1.7
Ala	30.9	3.5	2.8
Val	52.9	8.3	7.7
Met	0.8	0.7	0.6
Ile	16.3	8.4	8.6
Leu	46.9	7.5	7.3
Tyr	19.7	5.0	5.0
Phe	19.6	2.2	2.1
Cys	15.7	5.5	6.2
	550.4	119.2	119.0

differences in the amounts of α -chain or light chain in the two pools were detected. Specific antisera confirmed these observations: pool B reacted with antisera to secretory piece, α - and light chain, whereas

pool A reacted only with antisera to α - and light chain.

It must be emphasized that under normal physiological conditions the two populations of sIgA behaved identically; they were eluted together from a DEAE-cellulose column on application of a salt gradient in the absence of urea (profile not shown), and therefore presumably both contained secretory-piece molecules. It was only under dissociating conditions (in the presence of urea or sodium dodecyl sulphate) that a difference between them was observed; they therefore only differ in their mode of attachment of secretory piece. The sIgA in pool A has secretory piece non-covalently bound, and so this was removed by urea or sodium dodecyl sulphate, thus allowing its separation (on both a molecular-size and charge basis) from the sIgA in pool B, which has secretory piece covalently attached to it.

To determine whether these two populations of sIgA correlated in any way with the two serologically defined subclasses of rabbit α -chain (Hanly *et al.*, 1973), samples of pools A and B from a single rabbit (PF3) were typed (by Dr. K. L. Knight and Dr. C. W. Hanly) for the presence of f and g markers. Allotypic determinants f72, g74, f71 and g75 were detected in pool B but only g74 and g75 in pool A. Although these data were not quantitative, it appears that only molecules with g markers bind secretory piece non-covalently, whereas all of the molecules with f markers and some with g markers bind secretory piece covalently. Despite the fact that rabbit PF3 was heterozygous at both the f- and g-loci (f72 g74/f71 g75), the difference in the mode of attachment of secretory piece was related only to the subclass of the molecule and not to the allotypic variants within these subclasses.

Attempted isolation of α -chain

The major aim of the present study was to determine the amino acid sequence of the α -chain variable region; therefore the separation of α -chain from the other chains of sIgA was attempted. In these studies guanidine-treated sIgA (i.e. partially depleted of secretory piece) was used.

Initially, the separation was attempted after cleavage of only the interchain disulphide bonds (Prahl & Porter, 1968). Complete cleavage of the interchain bridges (as detected by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate) was only achieved at dithiothreitol concentrations of 50 mM or higher; this is 10 times the concentration required for reduction of IgG. Even after such treatment, it was not possible to separate the constituent chains by Sephadex G-100 chromatography by using various dissociating media, namely 1M-propionic acid, 6M-urea and 5M-guanidine hydrochloride.

Separation of the chains, after complete cleavage of all disulphide bonds, was then attempted (Cebra & Small, 1967). The standard procedure (O'Donnell *et al.*, 1970) involved reduction of sIgA (10 mg/ml) by 5.5 mM-dithiothreitol for 2 h, in 7M-guanidine hydrochloride, followed by [14 C]carboxymethylation and gel filtration on Sephadex G-100 in 5M-guanidine hydrochloride (Fig. 3). Polyacrylamide-gel electrophoresis, with and without sodium dodecyl sulphate, of samples from the peaks obtained (Fig. 4) showed that the first peak (H) contained both secretory piece and α -chain and the second peak (L) contained light chain. The third peak (J), which was detected in the column eluate only by its radioactivity, contained a very acidic band characteristic of J-chain (Halpern & Koshland, 1970). Amino acid analysis of this fraction (Table 1) on comparison with the composition of rabbit J-chain previously reported (Koshland, 1975) confirmed the view that the third peak was indeed J-chain. Of the A_{280} units recovered from this column, approx. 20% were present in the light-chain peak; the theoretical light-chain content of sIgA is 23% by weight (calculated from data presented in the introduction).

Three techniques were tried in attempts to resolve α -chain from secretory piece (first peak H, Fig. 3).

1. The mixture was passed down an affinity column consisting of antibodies against completely reduced and alkylated secretory piece coupled to Sepharose 4B (Cuatrecasas, 1970). In repeated experiments, 90% of the applied protein was bound to the column and was not eluted by 1M-propionic acid; the small amount of material not retained by the column still contained secretory piece.

2. The mixture was subjected to preparative electrophoresis in 5.6% (w/v) polyacrylamide gel, containing 6M-urea, by using the apparatus and buffer system of Brownstone (1969). Pure α -chain was

Fig. 3. Fractionation of completely reduced and [14 C]-carboxymethylated sIgA

Gel filtration was conducted on a column (3.2 cm $\times$ 140 cm) of Sephadex G-100 in 5M-guanidine hydrochloride. Fraction size was 10 ml. —, A_{280} ; ---, radioactivity (c.p.m./ml of eluate).

obtained (as judged by analytical polyacrylamide-gel electrophoresis), but only in 5–20% yield (by A_{280}); the remainder of the protein applied was found, on staining, at the point of application.

3. The mixture was treated with CNBr and the products were fractionated by gel filtration on Sephadex G-100 in 0.2M-sodium formate (pH 3.3)/6M-urea, in a manner analogous to that reported by Lamm & Greenberg (1972) for the purification of human secretory piece. The methionine content of secretory piece is less than 1 mol/mol of protein (Table 1), whereas the value for α -chain (calculated from amino acid analysis of the α -chain and secretory-piece mixture) is 1–2 mol/mol of protein; however, CNBr treatment of the mixture gave no reproducible smaller fragments.

The failure of all three techniques was attributed to aggregation of the protein chains caused by complete reduction and exposure to guanidine hydrochloride. Measurement of the degree of aggregation (see under 'Analytical methods') of the protein fractions obtained during the sIgA-isolation procedure supported this view (Table 2).

Limited proteolysis of sIgA: isolation of fragment $F(ab')_{2\alpha}$

Enzymic cleavage of the native sIgA molecule was performed in attempts to obtain an α -chain fragment

Fig. 4. Polyacrylamide-gel electrophoresis of the constituent chains of sIgA

Electrophoresis was performed in 5.6% (w/v) polyacrylamide gel in sodium dodecyl sulphate [(a) reduced and alkylated sIgA; (b) pool H from Fig. 3; (c) pool L from Fig. 3; (d) pool J from Fig. 3] or in 5.6% (w/v) polyacrylamide/alkaline-urea gel without sodium dodecyl sulphate [(e) reduced and alkylated sIgA; (f) pool H from Fig. 3; (g) pool L from Fig. 3; (h) pool J from Fig. 3].

Table 2. Degree of aggregation of protein fractions obtained during the sIgA-preparation procedure

Values were calculated from the relative amounts of protein excluded from, and partially included in Sepharose 6B beads in 0.1 M-sodium phosphate buffer, pH 6.8 (see under Analytical methods).

	Aggregated form (%)	Non-aggregated form (%)
sIgA after DEAE-cellulose chromatography	0	100
Freeze-dried sIgA after DEAE-cellulose chromatography	5–10	90–95
sIgA after gel filtration in 3 M-guanidine hydrochloride	30–40	60–70
α-Chain and secretory-piece mixture from completely reduced sIgA	100	0

containing the variable region. To avoid aggregation difficulties, the sIgA pool before guanidine treatment was used in all digests (Table 2).

Four enzymes were used for limited proteolysis (see the Experimental section): trypsin, 'IgA-protease', papain and pepsin. No cleavage of sIgA was detected by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate after digestion with trypsin or 'IgA-protease'. Papain yielded the fragment Fab_{2a} described by Hanly *et al.* (1973), but only in 20% yield of the amount digested.

In contrast, peptic digestion at pH 2.4 cleaved 80–90% of the sIgA molecules (Fig. 5). Specific antisera detected light-chain determinants in all of the fractions of the first two peaks; secretory-piece and α-chain determinants were found only in the

Fig. 5. Fractionation of a peptic digest of sIgA. Gel filtration was conducted on a column (5.0 cm × 90 cm) of Sephadex G-200 in 0.1 M-sodium phosphate, pH 6.8. Fraction size was 20 ml. Arrows indicate elution positions of marker proteins.

first peak and the early fractions of the second peak. The later fractions of the second peak [designated F(ab')_{2a} in Fig. 5] contained no intact sIgA, α-chain or secretory piece on polyacrylamide-gel electrophoresis in sodium dodecyl sulphate (Fig. 6), nor J-chain on alkaline-urea electrophoresis. After reduction and alkylation, fragment F(ab')_{2a} showed two major bands on gel electrophoresis (Fig. 6, gel e), one in the position of light chain and one of higher molecular weight.

Because of these properties, this pool was designated F(ab')_{2a} and considered to be homologous to the peptic fragment F(ab')₂ of IgG, which was characterized by Nisonoff & Dixon (1964). The molecular weight of fragment F(ab')_{2a} is approx. 110 000 from gel filtration and polyacrylamide-gel

Fig. 6. Polyacrylamide-gel electrophoresis of fragment $F(ab')_{2\alpha}$

Electrophoresis was performed in 5.6% (w/v) (a, b and c) or 10% (w/v) (d, e and f) polyacrylamide gel in sodium dodecyl sulphate (see under 'Analytical methods'): (a) sIgA; (b) $F(ab')_{2\alpha}$; (c) IgG; (d) completely reduced and alkylated sIgA; (e) completely reduced and alkylated $F(ab')_{2\alpha}$; (f) completely reduced and alkylated light chain.

electrophoresis (Figs. 5 and 6). The yield of fragment $F(ab')_{2\alpha}$ was 30–35% of the sIgA digested (by A_{280}).

It was also concluded from the analytical data that the first peak contained undigested sIgA, and that fragment $Fc_{2\alpha}$ and/or partially digested sIgA was present in the fractions between the first two peaks. The third peak (eluted in the void volume of the column) exhibited no detectable antigenic determinants and gave no bands on gel electrophoresis; this was assumed to consist of small peptides produced by complete degradation of portions of the sIgA molecule.

Therefore the $F(ab')_{2\alpha}$ preparation was free from J-chain, secretory piece and the Fc portion of α -chain; this preparation was used for *N*-terminal-sequence studies on the α -chain (Johnstone & Mole, 1975, 1977) and for isolation of fragment Fd_{α} .

Isolation of fragment Fd_{α} and *N*-terminal CNBr fragment of α -chain from fragment $F(ab')_{2\alpha}$

Partial reduction and alkylation of fragment $F(ab')_{2\alpha}$ at dithiothreitol concentrations of 0.1 M and higher split all of the interchain bonds, as determined by polyacrylamide-gel electrophoresis. However, gel filtration on Sephadex G-100 in various dissociating media (1 M-propionic acid, 5 M-guanidine hydrochloride) failed to separate the chains, presumably because of their similar molecular size (Fig. 5, gel e).

After complete reduction, alkylation and CNBr treatment of fragment $F(ab')_{2\alpha}$ (see the Experimental section), several fragments were resolved (Fig. 7); the first two peaks were recovered in approximately equal amounts as judged by radioactivity and A_{280} . The second peak contained light chain, as judged by

Fig. 7. Fractionation of completely reduced and $[^{14}C]$ -carboxymethylated $F(ab')_{2\alpha}$ after CNBr cleavage. Gel filtration was conducted on a column (3.2 cm $\times$ 140 cm) of Sephadex G-100 in 6 M-urea/0.2 M-sodium formate, pH 3.3. Fraction size was 10 ml. —, A_{280} ; ---, radioactivity (c.p.m./ml of eluate).

detection of the κ -chain marker peptide (see under 'Analytical methods'), but very little, if any, of this peptide was found in the first peak. It was concluded that the second peak (eluted in the position of completely reduced light chain) consisted mainly of light chain, and that the first peak (which was also excluded from Sephadex G-200 gel) contained mostly fragment Fd_{α} in a very aggregated condition.

The amino acid composition and yield of the third peak (elution volume 730 ml), which was detected in the column eluate only by its radioactivity, indicated that it was homologous to the *N*-terminal 34-residue fragment of γ -chain (Tables 3 and 4). Characterization of the $[^{14}C]$ carboxymethylcysteine residues (O'Donnell *et al.*, 1970) in this pool detected only the marker peptides of heavy-chain cysteine residue 22.

Homoserine was also detected, by amino acid analysis, in the trailing edge of the second (light-chain) peak (elution volume 530 ml) and this was taken to indicate the presence of the *N*-terminal 79-residue CNBr fragment of α -chain (by homology with γ -chain). However, because this pool was

Table 3. *Amino acid composition of the N-terminal 34-residue fragment of α - and γ -chain*

Compositions are given as mol of residue/mol of peptide. Data for the γ -chain fragments are taken from Wilkinson (1969a).

	Aa1 γ	Aa1 α	Aa3 γ	Aa3 α
Lys	0.0	0.4	1.0	0.8
Arg	1.0	1.0	0.2	0.4
Asp	0.7	1.7	1.6	2.0
Thr	5.1	4.4	2.9	3.1
Ser	5.1	4.4	4.9	4.3
Glu	3.0	3.2	3.9	4.0
Pro	2.4	2.1	1.4	2.0
Gly	4.6	4.2	4.8	4.3
Ala	0.9	1.1	1.7	1.9
Val	2.3	2.5	1.6	2.1
Ile	0.3	0.8	0.3	0.9
Leu	4.5	3.7	3.8	3.1
Tyr	1.0	1.0	1.4	1.2
Phe	1.0	0.7	1.6	1.3
CmCys	0.9	0.9	0.8	0.8
Hse	1.1	1.0	0.9	0.8
	33.9	33.1	32.8	33.0

Table 4. *Yield of the N-terminal 34-residue fragment from α - and γ -chain*

Yields are expressed as mol of fragment/mol of heavy chain. Data for the γ -chain are taken from Prahl & Porter (1968).

	γ -Chain		α -Chain
	Actual yield after two chromatography steps	Relative yield corrected for losses	Actual yield after one chromatography step
Aa1	0.37	0.57	0.50
Aa3	0.23	0.45	0.35

contaminated with light chain and in low yield (5–15%), it was not investigated further.

Discussion

The separation of two populations of sIgA supports Lawton *et al.* (1970), who postulated the existence of such populations from serological studies. Partial correlation of these populations with the subclasses of sIgA is in agreement with the data of Knight *et al.* (1975), who found that secretory piece was dissociated from molecules with g antigenic markers, but not from those with f markers on polyacrylamide-gel electrophoresis in sodium dodecyl sulphate. Knight *et al.* (1975) could not determine by this approach whether all of the secretory piece present on molecules

with g antigenic markers was removed under dissociating conditions. The present study shows that only a portion of these molecules binds secretory piece non-covalently, although it was not possible to determine the amount. Further serological and structural studies are needed, but the existence of non-allelic variants of the gene encoding molecules with g antigenic markers seems likely. The possibility that the differential susceptibility of the subclasses of sIgA to proteolytic cleavage (Knight *et al.*, 1973) is related to the way in which secretory piece is bound remains to be evaluated.

After reduction and alkylation of sIgA it was not possible to separate α -chain from secretory piece. Use of this impure preparation of α -chain in structural studies (Wilkinson, 1969b) has given misleading results (clarified by Johnstone & Mole, 1975).

The resistance of rabbit sIgA to enzymic cleavage is well documented (Steward, 1971; Lawton, 1971), and these observations were confirmed in this study. Trypsin and IgA-protease did not cleave rabbit sIgA, in contrast with their action on human IgA (Zikan *et al.*, 1972; Plaut *et al.*, 1974). Also, the F(ab')₂ fragment was obtained under conditions of peptic digestion that degrade rabbit IgG to small peptides (Haber & Stone, 1967). The present study also demonstrated the relative resistance of sIgA to chemical cleavage, and separation of the constituent chains was not possible even under dissociating conditions after reduction of interchain bonds. This property presumably reflects the need for sIgA to function under extreme conditions in the secretions.

The low yield of the papain fragments compared with the results of Hanly *et al.* (1973) can be explained by the fact that many of the rabbits used in our study were of the f71 g75 allotypes, and in such animals molecules with f antigenic markers, which are not susceptible to papain digestion (Knight *et al.*, 1973), comprise the majority of the sIgA pool (K. L. Knight, personal communication).

Digestion with pepsin, rather than with papain, was adopted as a preparative step for three reasons. Firstly, the yield of the peptic F(ab')₂ fragment was greater than that of the papain fragment. Secondly, portions of the sIgA molecule not wanted in our present sequence studies (Fc α and secretory piece) were mostly degraded to small peptides by pepsin. Thirdly, pepsin is irreversibly inactivated at neutral pH (Fruton, 1971), and so neutralization of the digest removed any possibility of an active proteinase remaining attached to fragment F(ab')₂ and affecting subsequent studies.

The yield of fragment F(ab')₂ (30–35% by weight of sIgA digested) is approximately half of the theoretical yield, the remainder being completely degraded. Whether this low yield reflects the differential susceptibilities of molecules with f and g antigenic markers to peptic digestion remains to be established.

Fleischman *et al.* (1963) described the separation of fragment Fd from light chain after partial reduction of rabbit fragment Fab, by virtue of the specific dimerization of the former in 1M-propionic acid. Although this phenomenon could not be demonstrated for fragment F(ab')_{2α}, the Fd_α fragment appeared to aggregate specifically in urea at low pH after complete reduction, and this allowed its separation from light chain in a manner analogous to the technique of Fleischman *et al.* (1963).

The isolation of fragments F(ab')_{2α} and Fd_α, and the α-chain N-terminal 34-residue fragment described in the present paper, allowed further structural characterization of the sIgA molecule. Primary-sequence studies on these fragments are reported in the following paper (Johnstone & Mole, 1977).

We thank Professor R. R. Porter and Miss E. M. Press for their advice and encouragement throughout this study, and their helpful discussion, together with that of Dr. A. F. Williams, in the preparation of this manuscript. We are most grateful to Dr. K. L. Knight and Dr. C. W. Hanly for detection of f and g markers in our sIgA preparations. Our thanks are also due to Mr. A. C. Willis for excellent technical assistance.

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