Papain Cleavage of Rabbit Secretory Immunoglobulin A. Differential Sensitivity of f and g Subclasses†

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ABSTRACT: Rabbit secretory immunoglobulin A (sIgA) was digested with papain in the absence of reducing agent and four fractions were isolated by gel filtration. Three of the four fractions, undigested sIgA, $Fc_{2\alpha}$, and $Fab_{2\alpha}$ have previously been characterized (Hanly *et al.* (1973), *Biochemistry 12*, 733); the fourth fraction (approximately 15% of the total protein) was identified as Fab_{α}-like material by its sedimentation and antigenic properties. Each of the four fractions was analyzed for the allotypic specificities controlled by the *f* and *g* loci by quantitative precipitation of the radiolabeled fractions with anti-f or anti-g antisera. The f allotypic specificities were found predominantly in the undigested sIgA fraction and the g allotypic specificities were found predominantly in the $Fc_{2\alpha}$ and $Fab_{2\alpha}$ fractions. The Fab_{α}-like fractions contained some molecules with f and some with g specificities. Papain digestion of individually purified f72, f73, or g74 sIgA molecules also showed that the f molecules are highly resistant to papain cleavage and that the g molecules are highly sensitive to proteolytic digestion by papain. Thus, allotypes representing the two subclasses of rabbit sIgA have differential sensitivities to digestion by papain.

Two subclasses (f and g) of rabbit IgA have been identified and have been shown to be controlled by two closely linked loci, *f* and *g*. Genetic variants of each locus have been found: three alleles, f^{71}, f^{72}, and f^{73}, of the *f* locus control the allotypic specificities f71, f72, and f73, respectively, and two alleles, g^{74} and g^{75}, of the g locus control the allotypic specificities g74 and g75, respectively. All individual rabbits have a population of IgA molecules with f allotypic specificities and still another population of IgA molecules with g allotypic specificities (Conway *et al.*, 1969a,b; Hanly *et al.*, 1973). Of the six possible linkage combinations between the alleles of the *f* and *g* loci, only three ($f^{71}g^{75}$, $f^{72}g^{74}$, and $f^{73}g^{74}$) have been found in the rabbit colony of S. Dray (University of Illinois Medical Center) (Conway *et al.*, 1969b). The *f* and *g* loci are closely linked to the *a* locus which controls the variable region of the heavy chains (V_H) of all rabbit immunoglobulin classes (Hanly *et al.*, 1972). Using an antiserum having both anti-f and anti-g activity we showed that the *f* and/or *g* allotypic specificities reside on the constant portion of α chains, *i.e.*, the anti-f,g reagents reacted with the $Fc_{2\alpha}$ and also with the $Fab_{2\alpha}$ fragments obtained by papain digestion of secretory immunoglobulin A (sIgA) (Hanly *et al.*, 1973). It was not clear, however, if both the f and the g determinants were present on the $Fc_{2\alpha}$ and $Fab_{2\alpha}$ fragments or whether only one set of determinants (f or g) was present. In an effort to clarify this problem, individually specific anti-f72, anti-f73, and anti-g74 antisera were obtained and were reacted with papain fragments of sIgA. We now report the results of these studies.

Materials and Methods

Papain Digestion. Secretory IgA was isolated from colostrum and milk of individual rabbits by the methods described previously (Cebra and Robbins, 1966; Hanly *et al.*, 1973). The sIgA obtained from Sephadex G-200 column chromatography of whey samples was further purified by filtration through a Bio-Gel P-300 column (2.5 × 150 cm) at room temperature in borate–saline buffer (0.2 M borate–0.15 M NaCl), pH 8.0. With the second filtration step, a small amount of high molecular weight material (less than 10% of the total protein) was eluted in front of the 11S sIgA peak. By immunoelectrophoresis, the purified 11S sIgA revealed a single precipitin arc when reacted with goat anti-(rabbit)whey. A 1–2% solution of purified 11S sIgA was reacted with cysteine-activated papain (2–4 mg of papain/100 mg of sIgA) for 4 hr at 37°, after which time the resulting fragments were separated by filtration through a Bio-Gel P-200 column (2.5 × 150 cm; room temperature) (Hanly *et al.*, 1973).

Antisera. Goat anti-(rabbit)sIgA, goat anti-(rabbit)α-chain, goat anti-(rabbit)secretory component (SC), and goat anti-(rabbit)Fc_{γ} were the same as previously described (Hanly *et al.*, 1973). Goat anti-(rabbit)Fc_{α} was prepared by the injection of a goat with specially purified pooled samples of $Fc_{2\alpha}$ obtained from papain digests of sIgA. The $Fc_{2\alpha}$ fragments, initially separated from the other fragments of the papain digests by gel filtration, were subjected to disc electrophoresis to remove traces of contaminating $Fab_{2\alpha}$. After electrophoresis, the portion of each gel containing $Fc_{2\alpha}$ was cut out, homogenized with a mortar and pestle, mixed with Freund's adjuvant and injected intramuscularly into the goat. The first injection contained approximately 1 mg of $Fc_{2\alpha}$ with Freund's complete adjuvant; the second injection, given 1 month later, contained 0.5 mg of $Fc_{2\alpha}$ with Freund's incomplete adjuvant. The goat was bled weekly after the second injection. The antiserum reacted with intact sIgA and with $Fc_{2\alpha}$, but not with $Fab_{2\alpha}$ as determined by immunoelectrophoretic and radioprecipitation studies.

Guinea pig anti-(goat)IgG was prepared by injecting each of 10 guinea pigs with 1 mg of goat IgG in Freund's complete adjuvant. The goat IgG used for immunization was isolated from serum by two precipitations with sodium sulfate (18 and 14%) (Kekwick, 1940) followed by DEAE-cellulose chromatography in 0.01 M phosphate (pH 7.5) (Levy and Sober,

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1960). The guinea pigs were injected in the footpads, intramuscularly in the thighs, and subcutaneously in the neck. One month after the primary injection, each guinea pig was injected with 1 mg of goat IgG in Freund's incomplete adjuvant and was bled weekly thereafter.

The anti-allotype antisera, anti-a1, anti-a2, anti-a3, anti-b4, anti-b5, anti-f73,g74, anti-f72,g74, anti-f73, and anti-f72 were prepared as previously described (Conway *et al.*, 1969a,b). Anti-g74 was prepared by the injection of a rabbit (IgA allotype f71,g75) with 1 mg of g74 sIgA in Freund's complete adjuvant. Donor and recipient allotypes controlled by the *a* and *b* loci were matched. The g74 sIgA was separated from f73,g74 sIgA by the use of an immunosorbant in which IgG from anti-f73 antiserum was coupled to Sepharose. One month after the first injection, the rabbit was injected with 1 mg of g74 sIgA mixed with Freund's incomplete adjuvant and was bled weekly thereafter.

Goat anti-(rabbit)light chain and goat anti-(rabbit)J chain were gifts from Dr. A. Nisonoff and Dr. M. Koshland, respectively.

Isolation of Individual Subclasses of sIgA. The f72, f73, and g74 sIgA molecules were separated from each other by the use of immunosorbant columns containing IgG from either anti-f73 or anti-g74 antiserum coupled to Sepharose 4B (Axen *et al.*, 1967; Wofsy and Burr, 1969). The f72 and the f73 sIgA samples were prepared by passing f72,g74 sIgA or f73,g74 sIgA, respectively, through an anti-g74 immunosorbant column. The g74 sIgA was bound to the immunosorbant, whereas the f72 or the f73 molecules, respectively, appeared in the effluent when the column was washed with borate–saline buffer (pH 8.0). The g74 sIgA could be eluted with 0.1 M glycine (sulfate), pH 2.3, but it was not used in these experiments due to the possible denaturing effects of the acid eluent. The g74 sIgA used in these experiments was prepared by passing f73,g74 sIgA through an anti-f73 immunosorbant column. The g74 sIgA appeared in the effluent when the column was washed with borate–saline buffer (pH 8.0).

Radioprecipitation Analyses. The samples used for radioprecipitation analyses were iodinated with ^{125}I either by the ICl method (McFarlane, 1958) or by the lactoperoxidase method (Marchalonis, 1969). Whole sIgA was iodinated by the lactoperoxidase method since this method appeared to cause less denaturation of sIgA than the ICl method as indicated by the percent of radioactivity precipitated by anti-sIgA. Radioprecipitation analyses were done by the methods previously described (Hanly *et al.*, 1973). In some experiments, the radioprecipitations with goat antisera (goat anti-α, goat anti-SC, goat anti-L chain, goat anti-J chain, or goat anti-Fc_α) were done using guinea pig anti-(goat)IgG as an indirect reagent. Five microliters of goat antiserum were added to the ^{125}I-labeled antigen, and after 1-hr incubation at 37°, sufficient guinea pig anti(goat)IgG was added to achieve slight antibody excess in order to assure precipitation of the complexes formed between the ^{125}I-labeled antigen and the goat antiserum.

Analytical Methods. Immunoelectrophoresis was performed in 1.5% Noble agar in 0.05 M sodium barbital buffer, pH 8.6 (Grabar and Williams, 1955). Disc electrophoresis was done at pH 8.9 in 7.5% polyacrylamide gels by the method of Williams and Reisfeld (1964). Disc gels were stained with Coomassie Blue (Weber and Osborn, 1969). Sedimentation velocity was determined with schlieren optics in the Spinco Model E ultracentrifuge in which the An-D rotor with 12-mm double-sector cells was used. Ultracentrifugation was done at 20° and 60,000 rpm; the protein samples were in borate–saline buffer (pH 8.0). The sedimentation coefficients reported are not corrected to zero concentration. Sedimentation equilibrium studies were done in a Spinco Model E ultracentrifuge with the use of a multiplex and ultraviolet scanner (Lamers *et al.*, 1963). The samples were centrifuged in borate–saline buffer (pH 8.0) at 12° with velocities of 12,000 rpm and 18,000 rpm. For molecular weight calculations of Fab_α, a $\bar{v}$ of 0.741 was used (Miller and Metzger, 1966).

Results

Characterization of an Fab-like Fragment from Papain Digests of sIgA. Papain digests of f73,g74 sIgA passed through a column of Bio-Gel P-200 separated into four peaks (Figure 1). The first three peaks, containing approximately 85–90% of the original material, have been characterized as undigested sIgA, $Fc_{2\alpha}$ and $Fab_{2\alpha}$, respectively (Hanly *et al.*, 1973). Ten to fifteen per cent of the protein material was eluted after the $Fab_{2\alpha}$ and is designated peak 4. The fractions of peak 4 were pooled, as indicated in Figure 1, and concentrated by ultrafiltration. Characterization studies of the protein of pool 4 were done with the following methods: ultracentrifugation, immunoelectrophoresis, disc electrophoresis, and quantitative radioimmunoprecipitation.

Analytical ultracentrifugation of pool 4 revealed a single symmetrical peak with a sedimentation coefficient of 3.2 S (not corrected for concentration). Sedimentation equilibrium studies were done on three samples of pool 4 obtained from papain digests of sIgA from three different rabbits. The molecular weight values as determined at 12,000 rpm and calculated with a $\bar{v}$ of 0.741, were 46,900, 48,200, and 49,900 daltons. The values as determined at 18,000 rpm agreed within 3% of the values determined at 12,000 rpm for each of the samples.

Pool 4 material subjected to disc electrophoresis migrated as a diffuse band with a variable number (0–7) of discrete bands distributed within the diffuse band (Figure 2). In some samples, only the diffuse band was present. The diffuse character of the band is similar to that of $Fab_{2\alpha}$ (Figure 2) but is in contrast to the discrete bands characteristic of $Fc_{2\alpha}$ and undigested sIgA (Hanly *et al.*, 1973). On immunoelectrophoresis, pool 4 formed an intense precipitin arc after reaction with anti-sIgA (Figure 3). This precipitin arc was similar in position and length to the precipitin arc formed by the reaction of $Fab_{2\alpha}$ and anti-sIgA (Figure 3). No precipitin reaction was observed when pool 4 material was reacted with anti-$Fc_{2\alpha}$. A second faint precipitin arc formed by the reaction of pool 4 and anti-sIgA is indicated by an arrow in Figure 3. This precipitin arc is similar in position to the precipitin arc formed by the reaction of pool 4 and anti-SC (Figure 3).

By radioprecipitation analyses, most of the ^{125}I-labeled protein of pool 4 was precipitated by the appropriate anti-a or anti-b reagents. Less than 8% of the radioactivity was precipitated by anti-Fc_α or by anti-SC (Table I). Since pool 4 material has: (1) a sedimentation coefficient of 3.2 S; (2) a molecular weight of approximately 48,000; (3) little or no Fc portion of the alpha chain; (4) less than 8% secretory component, and since most of the molecules have *a* heavy-chain (V_H region) and *b* light-chain specificities, pool 4 appears to contain Fab-like fragments.

Quantitative Analysis of Papain Fragments of sIgA Using Individually Specific Anti-allotype Antisera. Individually specific antisera, anti-g74, anti-f72, and anti-f73, were reacted with each of the three previously described fragments (undigested sIgA, $Fc_{2\alpha}$, $Fab_{2\alpha}$) and also with the Fab_α-like frag-

TABLE I: Per Cent Precipitation of ^{125}I-Labeled Pool 4 (Fab_{α}) of Papain-Digested sIgA from Ten Rabbits.

Rabbit	Allotype	Antibody
		Anti-a
J170-3	a1	76
H52-3	a1	76
H51-2	a1	71
H70-1	a2	78
G102-2	a3	38
G208-1	a2	77
H356-1	a2	73
H52-2	a1	85
F178-3	a1	70
J34-1	a1	50
		Anti-b
J170-3	b4	87
H52-3	b4,b5[a]	92
H51-2	b5	81
H70-1	b4	83
G102-2	b4	75
G208-1	b4	92
H356-1	b4	77
H52-2	b4,b5[a]	85
F178-3	b5	85
J34-1	b5	50
		Anti-Fc_{α}
J170-3		1
H52-3		2
H51-2		6[b]
H70-1		2
G102-2		1
G208-1		3
H356-1		1
H52-2		1
F178-3		1
J34-1		2
		Anti-SC
J170-3		1
H52-3		3
H51-2		5
H70-1		5
G102-2		5
G208-1		4
H356-1		3
H52-2		1
F178-3		4
J34-1		7

[a] Value represents sum of radioactivity precipitated by anti-b4 plus anti-b5. [b] Anti-α.

ment described above. Nearly all (76–94%) of the radioactivity in pool 1 (undigested sIgA) was precipitated by the anti-f reagents (anti-f72 and/or anti-f73) whereas 1–7% of the radioactivity was precipitated by anti-g74 (Table II). These data indicate that essentially all of the g74 sIgA molecules are digested by papain. Less than 6% of the radioactivity in the $Fab_{2\alpha}$ and $Fc_{2\alpha}$ peaks was precipitated by the anti-f reagents, whereas 86–95% of the radioactivity in the $Fab_{2\alpha}$ and 71–80% of the radioactivity in the $Fc_{2\alpha}$ was precipitated by anti-g74. These data also indicate that the g74 molecules are digested by papain and that few if any of the f72 or f73 sIgA

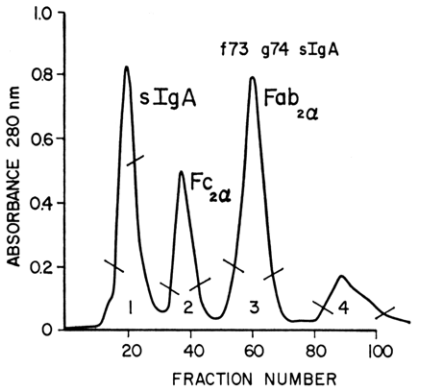

FIGURE 1: Elution pattern of the papain digest of sIgA from Bio-Gel P-200 (2.5 × 150 cm) in borate–saline buffer (pH 8.0). The sIgA is from rabbit H52-3 (al, b4, b5, f73, and g74).

molecules are digested into $Fc_{2\alpha}$ and $Fab_{2\alpha}$ fragments. Whereas, in general, the anti-a and the anti-b reagents each precipitated more than 70% of the radioactivity in pool 4 (Fab_{α}) (Table I), the anti-f,g reagents precipitated from 24 to 78% of the radioactivity (Table II). Most of the radioactivity of pool 4 precipitated by the anti-f,g reagents is due to the presence of the f72 and/or f73 specificities rather than g74 determinants (Table II). These data indicate that a small percentage of f72 and f73 sIgA molecules are digested by papain and that from these molecules an Fab_{α}-like product can be obtained.

Papain Digestion of Individually Purified f72, f73, and g74 sIgA. Individual samples of the f72, the f73, and the g74 sIgA were isolated from f72,g74 or f73,g74 sIgA by the use of immunosorbants and were iodinated with ^{125}I by the lactoperoxidase method. Radioprecipitation analyses of these purified sIgA fractions revealed that the f72 and the f73 preparations were

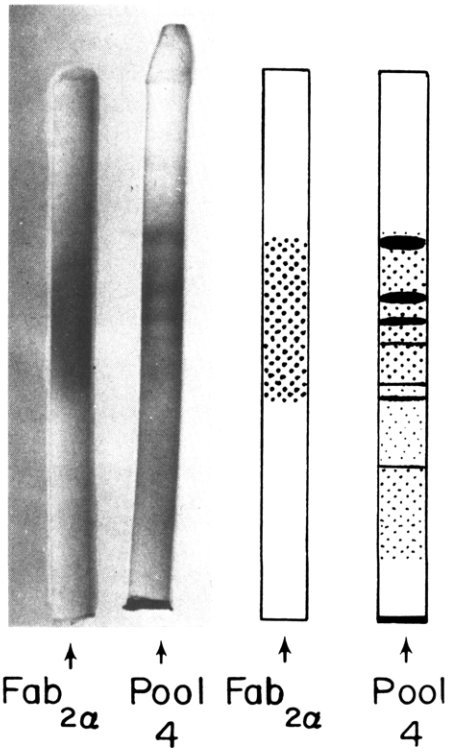

FIGURE 2: Disc electrophoresis in 7.5% polyacrylamide gel of the $Fab_{2\alpha}$ fragment and pool 4 from the papain digest of sIgA. The origin is at the top and the anode is at the bottom.

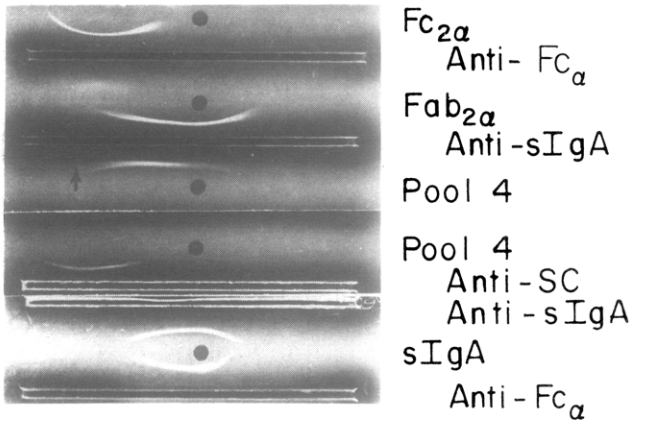

FIGURE 3: Immunoelectrophoresis in 1.5% Noble agar showing the precipitin reactions of pool 4 from papain digestion of sIgA with goat anti-sIgA and with anti-SC. Anode is at the left.

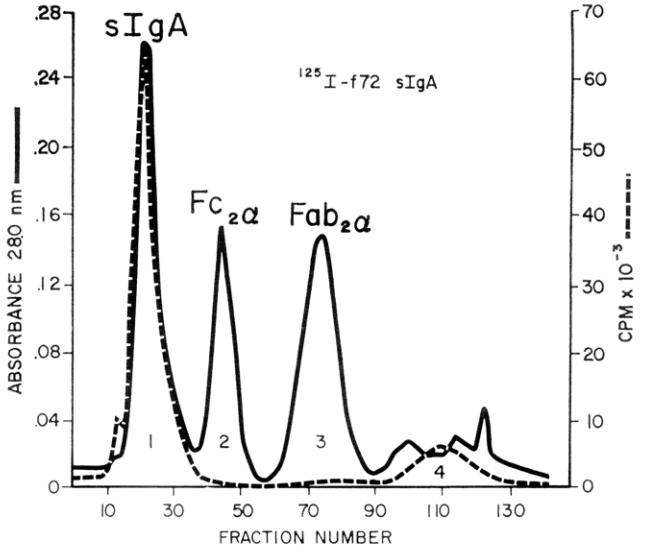

FIGURE 4: Elution pattern of the papain digest of sIgA from Bio-Gel P-200 (2.5 × 150 cm) in borate–saline buffer (pH 8.0). The sIgA used for digestion was a mixture of unlabeled f72,g74 sIgA and specifically purified ^{125}I-labeled f72 sIgA from rabbit J170-3.

essentially free of g74 molecules (less than 3%) and that the g74 preparations had a maximum of 10% contamination with f73 molecules. Each of these radioactively labeled sIgA samples was mixed with 10–20 mg of the parent sIgA, *i.e.*, the original unfractionated unlabeled f72,g74 or f73,g74 sIgA, and the mixture was digested with papain. The papain digests were then fractionated on Bio-Gel P-200 columns and the fractions obtained were monitored for radioactivity and for absorption at 280 nm (Figures 4–6). Nearly all of the radioactivity of the g74 sIgA molecules appeared in the $Fc_{2\alpha}$ and the $Fab_{2\alpha}$ fractions (Figure 6); less than 4% remained with undigested sIgA (pool 1). In contrast, approximately 75% of the f72 and f73 molecules were not digested by papain since most of the radioactivity (68 and 79%, respectively) appeared in the fraction containing undigested sIgA (pool 1). (Figures 4 and 5). These data show that essentially all of the g74 sIgA molecules are digested by papain and that most of the f72 and f73 sIgA molecules are resistant to cleavage by papain. Approximately 85% of the total radioactivity in the digested f72, f73, or g74 sIgA molecules was recovered in the four major peaks from the gel filtration column. Since the samples were not dialyzed after digestion with papain and before application to the gel filtration column, the f72, f73, and g74 sIgA molecules are not readily digested to dialyzable peptides.

Localization of J Chain. Radioprecipitation analyses revealed that 34–53% of the radioactivity of the sIgA molecules precipitated with goat anti-J chain (Table III). From 22 to 35% of the radioactivity in the undigested sIgA (pool 1) and approximately 40% (34–46%) of the $Fc_{2\alpha}$ fraction was precipitated by anti-J chain. Less than 3% of either the $Fab_{2\alpha}$ or the Fab_α-like fraction reacted with anti-J chain. Thus, J chain is probably associated with the Fc fragment of the sIgA molecule.

TABLE II: Per Cent Precipitation of ^{125}I-Labeled Fragments from Papain Digestion of sIgA from Six Rabbits.

Rabbit	Allotype	Pool 1	$Fc_{2\alpha}$	$Fab_{2\alpha}$	Fab_α
			Anti-f,g		
J170-3	f72,g74	91	78	98	24
J34-1	f72,g74	86	79	86	30
H51-2	f73,g74	93	81	97	78
H52-3	f73,g74	93	83	96	75
			Anti-f72		
J170-3	f72,g74	79	2	1	13
J34-1	f72,g74	76	1	1	18
H52-2	f72,f73,g74	32	2	1	6
F178-3	f72,f73,g74	34	1	1	7
			Anti-f73		
H51-2	f73,g74	94	1	5	64
H52-3	f73,g74	94	1	3	54
H52-2	f72,f73,g74	60	3	2	18
F178-3	f72,f73,g74	55	1	4	26
			Anti-g74		
J170-3	f72,g74	2	78	95	8
J34-1	f72,g74	7	74	92	4
H51-2	f73,g74	1	77	95	13
H52-3	f73,g74	1	80	92	15
H52-2	f72,f73,g74	1	71	86	1
F178-3	f72,f73,g74	1	80	92	5

Discussion

Five allotypic specificities (f71, f72, f73, g74, and g75) of rabbit IgA are controlled by two closely linked loci, *f* and *g*. The five specificities reside on separate IgA molecules and are not present on IgG or IgM (Conway *et al.*, 1969a). The *f* and *g*

TABLE III: Per Cent Precipitation of ^{125}I-Labeled sIgA and Papain Fragments of sIgA from Six Rabbits with Goat anti-J Chain.

Rabbit	sIgA	Pool 1	$Fc_{2\alpha}$	$Fab_{2\alpha}$	Fab_α
F178-3	48	22	42	1	1
#7	43	35	34	1	1
J34-1	53	24	46	ND[a]	ND
H52-2	34	26	40	2	ND
G248-1	47	ND	ND	ND	ND
H192-6	49	ND	ND	ND	ND

[a] ND = not done.

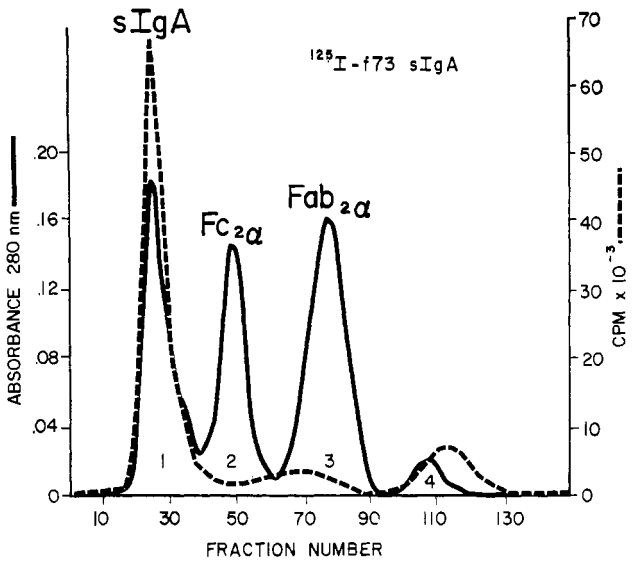

FIGURE 5: Elution pattern of papain digest of sIgA from Bio-Gel P-200 (2.5 × 150 cm) in borate–saline buffer (pH 8.0). The sIgA used for digestion was a mixture of unlabeled f73,g74 sIgA and specifically purified ^{125}I-labeled f73 sIgA from rabbit H52-3.

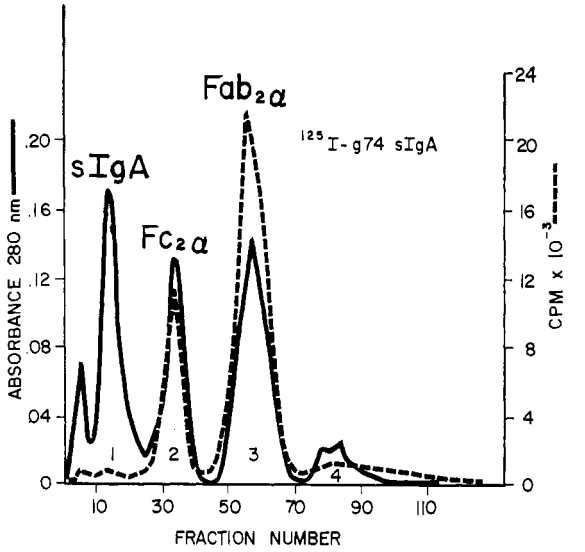

FIGURE 6: Elution pattern of the papain digest of sIgA from Bio-Gel P-200 (2.5 × 150 cm) in borate–saline buffer (pH 8.0). The sIgA used for digestion was a mixture of unlabeled f73,g74 sIgA and specifically purified ^{125}I-labeled g74 sIgA from rabbit H52-3.

loci are closely linked to the *a* locus which controls the variable portion of the Ig heavy chains, thus suggesting that the *f* and *g* allotypic specificities reside on the constant portion of the α chains (Hanly *et al.*, 1972). Previously we reported that doubly specific anti-f,g reagents reacted with the Fc$_{2\alpha}$ and also with the Fab$_{2\alpha}$ fragments obtained by papain digestion of sIgA but it was not clear whether the antisera were reacting with f and/or g allotypic determinants (Hanly *et al.*, 1973). Now with individually specific anti-f and anti-g reagents we have shown by quantitative radioprecipitation analyses that essentially all g74 molecules are cleaved by papain and that the Fab$_{2\alpha}$ and the Fc$_{2\alpha}$ fragments reacted only with anti-g74 and not with anti-f72 or anti-f73. Also, the undigested sIgA (pool 1) reacted only with anti-f72 and/or anti-f73 and not with anti-g74. Thus, all g74 molecules are cleaved by papain and most of the f72 and f73 molecules are resistant to cleavage by papain. Since anti-g74 reacted with essentially all of the molecules of the Fc$_{2\alpha}$ fraction and also with the Fab$_{2\alpha}$ fraction, it is evident that the g74 allotypic specificity is composed of multiple antigenic determinants distributed along the constant part of the alpha chain. Previous results by Ouchterlony analysis using anti-f73,g74 antiserum (Hanly *et al.*, 1973) suggested that the allotypic determinants present on the Fc$_{2\alpha}$ fragment were different from those on the Fab$_{2\alpha}$ fragment. Since only the anti-g74 (not anti-f72 or anti-f73) reacted with the Fab$_{2\alpha}$ and the Fc$_{2\alpha}$ fragment, it is likely that the g74 determinants present on the Fab$_{2\alpha}$ fragment differ from those on the Fc$_{2\alpha}$ fragment. Experiments are currently being done to characterize the g74 allotypic determinants present on the Fc$_{2\alpha}$ and on the Fab$_{2\alpha}$ fragments.

Proteolytic digestion of sIgA with papain results in three major peaks, previously identified as undigested sIgA, Fc$_{2\alpha}$, and Fab$_{2\alpha}$, and a minor peak containing less than 15% of the total protein (pool 4). That the latter peak contains Fab monomer-like fragments is indicated by the following characteristics of pool 4 material: (1) a sedimentation coefficient of approximately 3.2 S; (2) a molecular weight of approximately 48,000; (3) the presence of light chain determinants as shown by radioprecipitation analyses with anti-b reagents; (4) the presence of *a* allotypic determinants which reside in the variable portion of the heavy chain; and (5) the lack of determinants characteristic of the Fc portion of the heavy chain.

Results from radioprecipitation experiments indicate that the majority of the material in pool 4 is Fab$_\alpha$-like material, since in most experiments over 70% of the radioactivity of ^{125}I-labeled pool 4 material was precipitated by anti-a and by anti-b reagents. A greater variation was found in the amounts of radioactivity precipitated by anti-f,g reagents, *i.e.*, 24–78%. This discrepancy may be due to papain cleavage of different molecules at different sites thus giving rise to a heterogeneous group of Fab$_\alpha$-like fragments. In some molecules, papain may split N terminal to the allotypic determinants and in other molecules, it may split C terminal to at least one of the determinants. It has been previously shown that IgG may be cleaved by papain and pepsin at several sites (Smyth and Utsumi, 1967; Hill *et al.*, 1967).

Pool 4 material, however, is not homogeneous. The elution profile of peak 4 from the Bio-Gel P-200 column is somewhat variable and the peak is asymmetrical. The disc electrophoretic patterns of pool 4 material consistently revealed a diffuse band (similar to that of Fab$_{2\alpha}$) in addition to several discrete bands which varied in number, mobility and intensity from sample to sample. The observed variability of the disc electrophoretic patterns may reflect differences in selection of fractions of peak 4 for pooling, as well as differences in the degree of degradation. Secretory component and/or degradation products thereof, were present in a small but significant amount (up to 8%) as determined by radioprecipitation analyses. Immunoelectrophoretic analysis of pool 4 material also revealed a reaction between pool 4 material and anti-SC. The faint precipitin arc developed by anti-sIgA with electrophoresed pool 4 material is similar in position to that arc developed with anti-SC. The intense precipitin arc developed by the reaction of pool 4 with anti-sIgA is similar in position to that of Fab$_{2\alpha}$. Thus, although pool 4 contains predominantly Fab$_\alpha$-like material, it also contains other degradation products, one or more of which derives from secretory component.

Approximately 40% of the Fc$_{2\alpha}$ fragment is precipitated by anti-J chain, indicating that J chain may be attached to the

Fc portion of the α chain; this was previously suggested for α chain by Meinke and Spiegelberg (1951) and for μ chain by Mestecky *et al.* (1971). No J chain was found associated with the $Fab_{2\alpha}$ or Fab-like fragments of the molecule. As only 34–53% of intact 11S sIgA molecules are precipitated by anti-J chain, it would appear that either not all molecules contain J chain, or that if all molecules do have J chain, the determinants in some molecules are hidden. However, susceptibility of sIgA to cleavage by papain does not appear to be influenced by J chain since the proportion of molecules having J chain is not significantly different between intact sIgA, undigested sIgA (pool 1) and digested sIgA ($Fc_{2\alpha}$).

Whereas nearly all of the molecules of the g subclass were digested by papain, approximately 15% of the molecules of the f subclass were digested under the same conditions. It is clear that human IgG subclasses differ in their sensitivity to papain digestion (Virella and Parkhouse, 1971). It is also generally agreed that human IgA molecules differ in their sensitivity to proteolytic digestion (Tomasi, 1972); however, the nature of the molecules which are resistant (*e.g.*, different subclasses, or serum IgA *vs.* sIgA) and those which are sensitive to proteolytic digestion is not agreed upon. Results from our studies of rabbit sIgA are similar to those of Shuster (1971) in that he reported human IgA1 myeloma proteins were resistant to peptic digestion, whereas IgA2 myeloma proteins (Am2+ type) were highly susceptible. Moreover, BALB/c mouse IgA myeloma proteins, which resemble human IgA2 (Am2+) molecules, in that they lack light–heavy interchain disulfide bonds (Abel and Grey, 1968; Jerry *et al.*, 1970) are susceptible to proteolytic digestion (Grey *et al.*, 1970). While it is tempting to conclude that susceptibility to proteolytic digestion is a characteristic of the subclass, final verification must await chemical studies to determine the sites of enzymatic cleavage and the importance of the structural features of the allotypes. Since our present studies do not include the rabbit f71 and g75 molecules (as individually specific anti-f71 and anti-g75 are not yet available), we cannot rule out the possibility that the resistance and susceptibility to papain digestion are characteristics of particular allotypes rather than general characteristics of the f and g subclasses of rabbit sIgA. For example, chemical and immunochemical studies on human IgA have shown that disulfide bonded light chain dimers are a characteristic of the Am2+ allotype rather than a general structural feature of the IgA2 subclass (Jerry *et al.*, 1970; Mihaesco *et al.*, 1971).

The observation that digestion of g molecules results in Fab dimers and that the limited amount of digestion of f molecules results in Fab monomers may be explained in terms of disulfide bonding in one of three ways: (1) papain may cleave the α chain C terminal to an inter-heavy-chain disulfide bond in the g molecules and N-terminal to a comparable disulfide bond in the f molecules; (2) papain cleavage sites are similar in the f and the g molecules, but f molecules may not have an inter-heavy-chain disulfide bond in the N-terminal half of the α chain; or (3) neither the f nor the g molecules has an inter-heavy-chain disulfide bond N terminal to the site of papain cleavage. In the latter case, the g74 rabbit IgA molecules would resemble human IgA2 Am2+ type molecules (Jerry *et al.*, 1970; Wolfenstein-Todel *et al.*, 1972) or mouse BALB/c IgA molecules (Grey *et al.*, 1968; Warner and Marchalonis, 1972) in that their light chains are disulfide bonded to each other; the rabbit f subclass molecules would resemble human IgA1 molecules, *i.e.*, presence of L–H disulfide bonds. Thus papain digestion of rabbit g74 IgA in the absence of reducing agent would produce Fab dimer as a result of inter-light-chain disulfide bonding in these fragments, whereas digestion of f molecules would result in Fab monomer fragments. The relationship between human and rabbit subclasses and allotypes requires a determination of the type of light–heavy-chain bonding in rabbit IgA. This is not easily determined for secretory IgA as shown by Jerry *et al.* (1972) who found that secretory component could stabilize the potentially dissociable light chains of human IgA2 Am2+ type or of the pepsin $(Fab')_{2\alpha}$ fragment thereof. Adequate samples of rabbit IgA lacking secretory component (*e.g.*, serum IgA) are not readily available.

Additional structural information regarding IgA subclasses and allotypes and their interactions with secretory component and J chain should clarify the structural features related to proteolytic digestability and the resultant products. It will be important to determine if the population of IgA molecules resistant to proteolytic digestion serves in a different biological capacity than the population readily susceptible to proteolytic digestion.

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Purification and Characterization of Nascent Chains from Immunoglobulin Producing Cells†

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ABSTRACT: A method is described for preparing immunoglobulin nascent chains from mouse myeloma cells. Nascent chains are isolated by ion-exchange chromatography based on the strongly acidic charge conferred to peptidyl-tRNA by its nucleic acid moiety. The method is rapid, simple, gives essentially quantitative recoveries, and permits the subsequent serological isolation of a single protein species. Peptidyl-tRNAs isolated in this way have chromatographic properties which are sensitive to alkali and ribonuclease. They form a rapidly turning over pool on ribosomes, are released upon puromycin treatment, turnover is blocked by actidione, and they have a disperse size profile on sodium dodecyl sulfate–acrylamide gel electrophoresis. An immunoglobulin fraction devoid of tRNA is also present on ribosomes and some of its unusual chemical, kinetic, and physical characteristics are also described.

The study of early events in immunoglobulin biosynthesis by mouse myeloma cells requires a reliable method of preparing nascent polypeptide chains in good yields, uncontaminated by other cell proteins, and identifying them by specific antisera.

Currently used techniques range from one based on assuming that after short-term labeling all protein radioactivity in the polysome region of a sucrose gradient is nascent chains, to more elaborate procedures of degradation and analysis of the polysomal complexes. However, ribosome preparations are usually contaminated by nonribosomal components, and we show that such contamination confuses kinetics of protein synthesis even for short labeling periods. Methods that recover nascent chains after ribonuclease attack or, alternatively, salt precipitation of the nucleic acids and subsequent recovery of the protein components give poor yields of a mixture of nascent polypeptides with ribosomal proteins and possible other ribosome contaminants. A more selective approach is the release of nascent proteins from isolated polysomes by addition of puromycin *in vitro*. This has the disadvantage of inconsistent and generally low recoveries.

To avoid these difficulties, we adapted to myeloma cells a method devised for isolating nascent protein chains from bacterial lysates (Ganoza and Nakamoto, 1966) based on ion-exchange chromatography at pH 4.7 of peptidyl-tRNAs *via* the negative charge of the RNA moiety at this pH. The method is easily adapted to the analysis of myeloma cells, is rapid, and permits a quantitative recovery of nascent chains. Subsequent serological methods allow isolation of a single protein species (a light-chain immunoglobulin in this study). Chemical, kinetic, biological, and physical characteristics of the nascent chain fractions thus isolated are in accord with its identification as peptidyl-tRNA. Other proteins, associated with ribosomes, but not identifiable as nascent chains, were also investigated.

In the accompanying paper (Cioli and Lennox, 1973), these methods of isolating and identifying nascent chains are used to determine whether light chain is synthesized on membrane-bound or free ribosomes.

Materials and Methods

A flow sheet of the procedures is given in Figure 1.

Cells and Cell Suspensions. MOPC-46 tumors producing a K-type light chain (Melchers *et al.*, 1966) were maintained by subcutaneous transfer in female Balb/c mice and used 2–3 weeks after transfer. Transplant generations 42–51 were used for this study. Cell suspensions from freshly excised tumors were prepared as described in Choi *et al.* (1971a) in Eagle's medium (Dulbecco modified) without leucine and supplemented with 2.5% undialyzed horse serum and freshly dissolved glutamine. Cell densities between 1 and 5 $\times$ 10^7 cells/ml were used for the short incubations (1–5 min) and between 2 and 8 $\times$ 10^6 cells/ml for longer incubations.

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