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Proteolytic Fragmentation of Elasmobranch Immunoglobulins*

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ABSTRACT: Lemon shark 7S IgM, when digested by papain or trypsin, yielded a 6S (mol wt ~112,000) product plus small peptides. Upon reduction, the 6S fragment was cleaved into two 4.5S fragments (mol wt ~60,000). Analysis of heavy and light chains of the proteolytic products (either 6S or 4.5S) showed the light chains to be intact (mol wt ~22,000), but the heavy chain reduced in size from 70,000 to 43,000. Antigenic analysis of this heavy-chain fragment showed it to be missing some of the determinants of the intact heavy chain and hexose determinations showed it to have lost ~20% of its carbohydrate. It was concluded from these experiments that lemon shark 7S IgM can be cleaved by proteolysis to yield a 6S $F(ab\mu')_2$, which upon reduction yields two 4.5S fragments. Reduction and subsequent proteolysis of lemon shark 7S IgM, or proteolysis of 7S IgM subunits produced by reduction of lemon shark 19S IgM, yielded 3.5S ($Fab\mu$) material closely resembling the 4.5S fragment. This was considered to be the "core" of these immunoglobulin molecules. In these fragments light chains remained intact and the heavy chain was reduced to approximately light-chain size. Antigenic analysis showed the 3.5S fragment to be deficient in heavy-chain determinants to the 4.5S molecule and carbohydrate analysis showed 80% reduction in hexose content.

The surge of interest, during recent years, in the phylogenetic development of immunological competency has resulted in the demonstration that representatives of all major classes of vertebrates are capable of producing humoral antibodies in response to antigenic stimulation (reviewed, Clem and Leslie, 1969). However, in order to understand the mechanisms operative in the evolution of antibody structure and function, it is necessary to thoroughly characterize the immunoglobulins of various lower vertebrates and to relate these proteins to the immunoglobulins of mammals. Initial efforts in this direction from several laboratories have focused on the elasmobranchs and have resulted in the demonstration that dogfish (Marchalonis and Edelman, 1965), lemon (Clem and Small, 1967), nurse (Clem *et al.*, 1967), and leopard (Suran *et al.*, 1967) sharks each have 19S and 7S immunoglobulins. The shark 19S immunoglobulin is a pentamer of disulfide-linked subunits, each composed of two heavy (mol wt ~70,000) and two light (mol wt ~22,000) polypeptide chains, containing a relatively high carbohydrate content. These structural studies suggest that the shark 19S immunoglobulin belongs to the IgM class as defined for higher animals (World Health Organization, 1964). Based upon immuno- and physicochemical studies, the shark 7S immunoglobulin appears to belong to the same immunoglobulin class as the shark 19S molecule and therefore can be referred to as 7S IgM. The other immunoglobulin classes commonly found in mammals appear to be absent in sharks.

With the realization that enzymatic digestion of macromolecules may elucidate gross structural details, *i.e.*, differentiate between loosely structured regions (exceedingly enzyme susceptible) and highly structured regions such as helical areas (enzyme resistant) (Mihalya and Harrington, 1959), several investigators have studied the products of proteolysis of mammalian IgM (Miller and Metzger, 1966; Onoue *et al.*, 1966; Onoue *et al.*, 1968a; Mihaesco and Seligmann, 1967; Inman and Hazen, 1968; Goodman and Inman, 1969; Chen *et al.*, 1969; Beale, 1969; Suzuki, 1969). IgM fragments corresponding to IgG fragments [$Fc\mu$, $F(ab\mu')_2$, and $Fab\mu$] have been demonstrated to occur, and the polypeptide chain arrangement of IgG and IgM is thus probably quite similar.

With this high degree of structural similarity between different mammalian immunoglobulin classes and the gross similarity of lower vertebrate immunoglobulins to one of the

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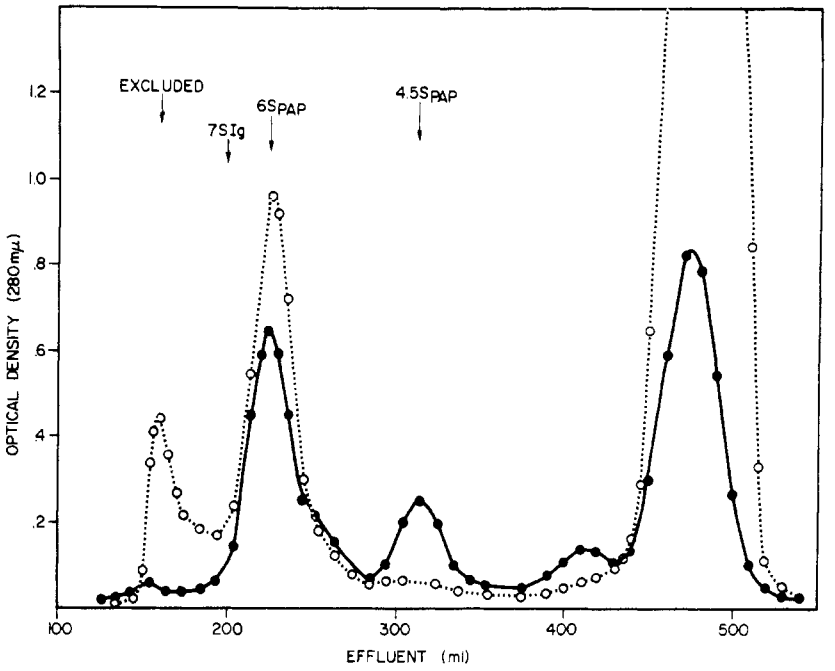

FIGURE 1: Sephadex G-200 gel filtration of lemon shark $7S_{NAT}$ after 30 min (○---○) and 9 hr (●—●) of papain digestion. Recovery of the 9-hr digest is ~84% with 37% of the material in the 6S peak, 12% as 4.5S material, and 58% as small peptides. Total recovery of the 30-min digest could not be calculated due to the ultraviolet absorbancy of iodoacetamide, but approximately 14% of the protein optical density is excluded and 35% appears as 6S material.

mammalian classes, it was decided to study the effects of proteolytic enzymes on shark immunoglobulins. This paper describes the enzymatic fragmentation of lemon shark 7S immunoglobulin and the reductive subunit of the 19S immunoglobulin with papain and trypsin.

Experimental Section

Preparation of Lemon Shark Immunoglobulins. Lemon shark 19S and 7S immunoglobulins were purified as described previously from whole serum by DEAE-cellulose chromatography and Sephadex G-200 gel filtration (Clem and Small, 1967). These two native proteins will be hereafter referred to as $19S_{NAT}$ and $7S_{NAT}$ for identification purposes.

Partial reduction of lemon shark immunoglobulins with dithioerythritol followed by Sephadex G-200 gel filtration was performed as discussed previously (Clem and Small, 1967). The "7S" regions from these columns were designated as $19S_{RED}$ (reductive subunit of $19S_{NAT}$) and $7S_{RED}$ (reductive form of $7S_{NAT}$); each of these reductive forms exhibited an $s_{20,w}$ of ~7 S and were antigenically identical with each other and with the unreduced proteins (Clem and Small, 1967). All shark immunoglobulins used in this study were checked for homogeneity at concentrations of ~8 mg/ml in the analytical ultracentrifuge and by immunoelectrophoresis employing rabbit antisera to whole lemon shark serum. Stock solutions of these lemon shark immunoglobulins and their reductive forms were stored at −20° at concentrations of 10–20 mg/ml in 0.14 M NaCl buffered at pH 7.4 with 0.015 M Tris-HCl until used. The concentrations of shark immunoglobulins were estimated using $\epsilon^{1\,cm}_{280m\mu,\,1\%} = 13.7$ (Clem and Small, 1967).

Proteolysis and Kinetic Studies. Digestion of proteins with papain (Worthington Biochemical Corp., Freehold, N. J.) was performed at 37° and pH 8.6 in either 0.15 M or 0.25 M NaCl, each containing 0.01 M cysteine and 0.002 M EDTA in the fashion of Porter (Porter, 1959). Kinetic studies were performed in unbuffered NaCl and the pH was kept constant by means of the addition of NaOH (0.05–0.1 M). In studies not requiring kinetic evaluation, pH was maintained with phosphate or Tris-HCl buffered NaCl. Enzyme was added to a level of 1% of the total protein. Proteolysis was stopped by the addition of 0.05 M iodoacetamide.

Tryptic hydrolysis was performed following the same general protocol used in the papain digestion experiments. A 1:100 enzyme (trypsin, three-times crystallized, Worthington Biochemical Corp.) to substrate ratio was established and the temperature was maintained at 37°. The pH for these studies was 8.2, again buffered by means of Tris-HCl or a phosphate system, except in kinetic studies where unbuffered NaCl was employed (0.15–0.25 M) and NaOH was continuously added to maintain the pH. In place of either cysteine or EDTA, 0.01 M $CaCl_2$ was added to the reaction vessel prior to adding trypsin (Miller and Metzger, 1966). Tryptic digestions were halted by the addition of equimolar amounts of soybean trypsin inhibitor (Worthington Biochemical Corp.) (Kunitz, 1947).

Digestions were performed in a recording pH-Stat (Radiometer, London Company, Westlake, Ohio) equipped with a 37° constant-temperature reaction flask. All work was performed under an atmosphere of N_2 gas which had been bubbled through 0.2 M H_2SO_4 and then distilled H_2O. For most studies, 20–50 mg of protein was digested in 3 ml of 0.25 M NaCl and titrated with the NaOH (0.05 or 0.1 M in distilled H_2O). A CO_2 trap was placed between the room air and the NaOH reservoir to eliminate the possibility of H_2CO_3 formation in the alkaline solution. A minimum of 1 hr was used to establish a baseline before the enzyme was added. When it appeared that the reaction was over, another 1% of enzyme was added to the reaction to be sure that lack of enzyme was not the limiting factor in these cases.

Miscellaneous. Antigenic analysis employing rabbit antisera,

sedimentation velocity studies, the determination of molecular weights by sedimentation equilibrium, and hexose determinations were all performed as described for previous studies on lemon shark immunoglobulins (Clem and Small, 1967). The estimation of polypeptide chain molecular weights by analytic gel filtration in the presence of 5 M guanidine·HCl was also performed as done previously (Clem and Small, 1967) except that the runs discussed here included simultaneously filtered standards of known molecular weights labeled with radioiodine.

Results

Papain Digestion of Lemon Shark 7S Immunoglobulin. Lemon shark $7S_{NAT}$ was digested with papain for varying periods of time and then subjected to Sephadex G-200 gel filtration. Figure 1 shows the elution profile (optical density at 280 mμ) of a sample digested for 30 min. This material no longer has the elution profile of the starting material, but now elutes in essentially three places. The first peak is excluded from the column and there appears to be a greater percentage of this peak after 30 min than at any other time studied. This observation has not been pursued further.

The second eluted peak had an $s_{20,w}$ of 6.1 S at a concentration of 6.3 mg/ml (referred to as $6S_{Pap}$), as determined in the Model E analytical ultracentrifuge. The last peak was where low molecular weight substances elute from this column. This peak was larger here than in the other optical density profiles because the reaction was stopped by the addition of iodoacetamide (which also absorbs ultraviolet light at 280 mμ) to inactivate the papain.

At the end of 9 hr of digestion, the Sephadex G-200 elution profile showed another peak becoming prominent concomitant with a decrease in the recovery of $6S_{Pap}$. Analytical ultracentrifugation showed it to contain material with an $s_{20,w}$ of 4.5 S at a concentration of 5.1 mg/ml (to be referred to as $4.5S_{Pap}$). It should be noted that the small molecule peak in this 9-hr digestion profile contained no alkylating agent and therefore presumably represented fragments of the immunoglobulin molecule digested by papain.

It was consistently noted that recovery of 6S fragments appeared quite low. For example, in going from $7S_{NAT}$ to $6S_{Pap}$, 60% of the mass of the molecule was retained (180,000 daltons digested to 110,000). However, only 30–40% of the optical density was retained in the 6S peak. This could have been due to either total digestion of some of the molecules or to a change in the extinction coefficient of the 6S molecule due to digestion of a portion of the immunoglobulin molecule rich in aromatic residues. It remains to be shown which of these two possibilities was responsible for the apparent low yield of $6S_{Pap}$, but future studies requiring amino acid composition data on the shark molecules may help to answer this question.

Immunoelectrophoretic studies and Ouchterlony analysis of the papain fragments were performed in order to study the relationships between intact $7S_{NAT}$ and the fragments. The electrophoretic mobility of both the $6S_{Pap}$ and $4.5S_{Pap}$ fragments did not change appreciably from that of the undigested material. There was a reaction of identity (Figure 2A) between the $6S_{Pap}$ and $4,5S_{Pap}$ fragments, and both these fragments showed partial identity with intact $7S_{NAT}$ and also $19S_{NAT}$. The antiserum utilized here was rabbit antiserum to lemon shark immunoglobulin. When the same material was analyzed with rabbit antisera to lemon shark heavy chains, as Figure 2B depicts, the antigenic deficiency of the $6S_{Pap}$ was again evident.

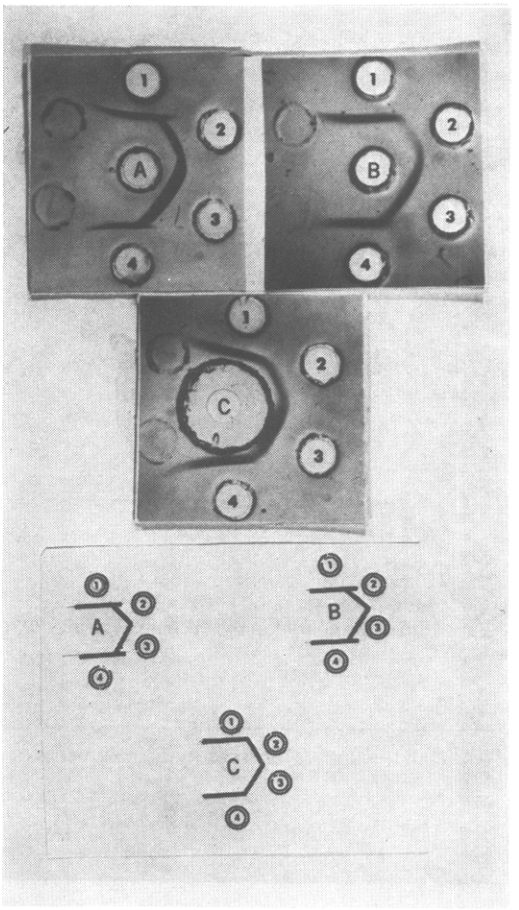

FIGURE 2: Ouchterlony analysis of lemon shark $7S_{NAT}$ and its proteolytic fragments. Concentrations of immunoglobulins and their fragments are ~1 mg/ml. Numbered wells contain: 1, $7S_{NAT}$; 2, $6S_{Pap}$; 3, $4.5S_{Pap}$; 4, $19S_{NAT}$. Lettered wells are: A, antiserum to $7S_{NAT}$; B, antiserum to $7S_{NAT}$ H chain; C, antiserum to $7S_{NAT}$ L chain.

However, as seen in Figure 2C, a reaction of identity between the fragments and both species of intact immunoglobulin was observed when rabbit antisera to lemon shark light chains was used to develop the precipitin bands.

Partial reduction (RED) of the $6S_{Pap}$ material with 0.1 M dithioerythritol and subsequent alkylation with an excess of iodoacetamide resulted in the breakdown of the molecule into $4.5S_{Pap,RED}$ subunits, antigenically identical with $6S_{Pap}$. It therefore appeared that the 4.5S protein obtained during papain digestion was merely the result of reduction of the 6S molecule by the cysteine utilized to activate the papain, so that both $4.5S_{Pap}$ and $4.5S_{Pap,RED}$ will be referred to subsequently as $4.5S_{RED}$.

Molecular weights of both $6S_{Pap}$ and $4.5S_{RED}$ were determined by sedimentation equilibrium. Plots of the logarithm of concentration (displacement of fringes from the depleted meniscus of the cell) *vs.* the square of the distance from the center of rotation were linear for each protein. These plots were made at different concentrations of protein and under different gravitational forces. Molecular weights (Table I) were calculated from the slopes of these lines. The weight-average molecular weight of $6S_{Pap}$ was calculated as ~110,000 while that of $4.5S_{RED}$ was ~60,000.

Papain digestion appeared to cleave a section of the heavy chain and thereby reduced the size of the 7S immunoglobulin molecule (molecular weight ~180,000) to 6S (molecular

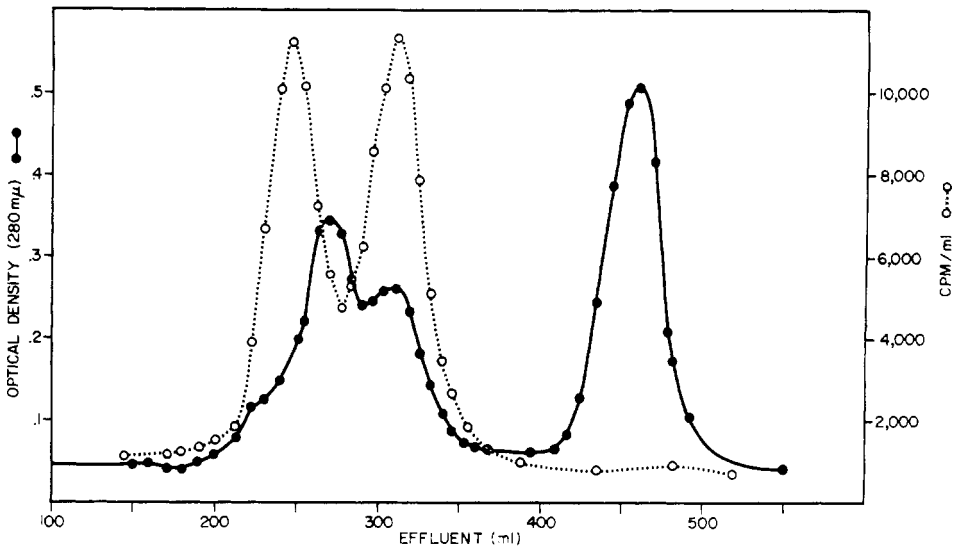

FIGURE 3: Sephadex G-200 (equilibrated in 5 M guanidine·HCl) fractionation of extensively reduced and alkylated lemon shark 6 S_{Pap} and an "internal standard" of 80 μg of ^{125}I-labeled rabbit IgG. V_0 = 175 ml and V_i = 306 ml for this column. Approximately 61% of the recovered OD_{280} (due to protein) is in the H chain peak and 39% is due to L chain.

TABLE I: Molecular Weights of Proteolytic Fragments of Lemon Shark 7S IgM.

		Concn (mg/ml)		
	rpm	0.25	0.5	0.75
$6S_{Pap}$	13,410	112,000 ± 500[a]	112,000 ± 1000	111,000 ± 500
	16,200	105,000 ± 1000	104,000 ± 4300	113,000 ± 600
$4.5S_{RED}$	25,980	59,000 ± 2500		
	21,740	62,000 ± 1900		
$3.5S_{Try}$	23,150	50,000 ± 1400	55,000 ± 4500	46,000 ± 1000
	25,980	49,000 ± 600	45,000 ± 700	48,000 ± 300

[a] These numbers represent the standard deviation of the least-squares line through the experimental points.

weight ~110,000). To further demonstrate this point, namely cleavage of the heavy chain, the following experiment was performed. A sample (25 mg) of $6S_{Pap}$ was extensively reduced and alkylated in 7 M guanidine·HCl along with 80 μg of ^{125}I-labeled rabbit IgG (as an internal standard) and passed through a calibrated Sephadex G-200 column equilibrated in 5 M guanidine·HCl. The elution profile (both optical density and radioactivity) of this column is shown in Figure 3. The K_D (0.4411) of the $6S_{Pap}$ light chain was indicative of a molecular weight of ~22,000. The lemon shark $6S_{Sap}$ heavy chains eluted with a K_D (0.3104) indicative of a protein with a molecular weight of ~43,000. This was considerably lower than the value of ~70,000 obtained earlier with lemon shark 7S IgM heavy chains (Clem and Small, 1967).

The fact that there were no apparent peptide bonds broken in either the 6S heavy (mol wt 43,000) or light (mol wt = 22,000) chains (each remained intact in the presence of 5 M guanidine·HCl and virtually 100% of the optical density was recovered as chains) suggested that two 4.5S fragments (molecular weight ~60,000) were produced from each reduced 6S fragment (molecular weight ~110,000). The data from molecular weight studies of both $6S_{Pap}$ and its constituent polypeptide chains coupled with antigenic comparisons of the intact 7S molecule and its papain fragments showed that papain digestion of $7S_{NAT}$ cleaves the H chain yielding a 43,000 mol wt fragment (as opposed to the 70,000 mol wt H chain of the native molecule) but leaves the L chain intact.

Trypsin Digestion of Lemon Shark 7S Immunoglobulin. Lemon shark 7S Ig was digested for 30 min and for 8 hr with trypsin and then subjected to Sephadex G-200 gel filtration. It was apparent from the elution profile of these columns that after 30 min the majority of $7S_{NAT}$ had been fragmented to lower weight material, although there appeared to be both a portion of the original immunoglobulin remaining and also possibly a variety of intermediate sizes. Specifically, ~63% of the starting OD_{280} was recovered and ~43% of this was eluted as 6S material. At the end of 8 hr of digestion, there was no longer any detectable $7S_{NAT}$ and the remaining material had an $s_{20,w}$ comparable to that of $6S_{Pap}$. This molecule will subsequently be referred to as $6S_{Try}$. The elution profile at this time showed ~66% recovery of the starting OD_{280} with ~88% of this as 6S material.

Both Ouchterlony and immunoelectrophoretic studies were

used to characterize the products of tryptic digestion of the $7S_{NAT}$. $6S_{Try}$ and $6S_{Pap}$ were antigenically identical with each other and deficient with respect to intact lemon shark immunoglobulin. Immunoelectrophoretic studies indicated that $6S_{Try}$ had not changed appreciably in mobility from $7S_{NAT}$. Partial reduction of $6S_{Try}$ resulted in the production of $4.5S_{Try}$ subunits which were antigenically identical with $6S_{Pap}$ and $4.5S_{RED}$.

Again, a sample (10 mg) of $6S_{Try}$ was extensively reduced and alkylated in the presence of 7 M guanidine·HCl along with 80 μg of ^{125}I-labeled rabbit IgG, fractionated on a calibrated Sephadex G-200 column equilibrated in 5 M guanidine·HCl, and the K_D's of the eluted materials were calculated. The profile of this column was similar to that depicted in Figure 3 indicating the L chain molecular weight to be 22,000 and the $6S_{Try}$ shark heavy chains to be ~43,000 as with $6S_{Pap}$.

From this data, it appeared reasonable that $6S_{Try}$ and $4.5S_{Try}$ are comparable to $6S_{Pap}$ and $4.5S_{RED}$, respectively.

Papain Digestion of Partially Reduced and Alkylated Lemon Shark Immunoglobulins. As mentioned in the Experimental Section, partial reduction of both $19S_{NAT}$ and $7S_{NAT}$ resulted in the production of 7S molecules, indistinguishable from each other in many ways. In an effort to determine the consequences of reducing shark immunoglobulin molecules on susceptibility to proteolysis, samples of partially reduced immunoglobulins were digested with papain.

$19S_{RED}$ and $7S_{RED}$ were digested for 2 hr and subjected to Sephadex G-200 gel filtration. The profiles from these columns indicated two major peaks from each preparation. The largest (~70% of the recovered material) was a broad peak containing low molecular weight materials. The peak which eluted earlier (~20% of the recovered material) was found to have an $s_{20,w}$ of 3.4S at a concentration of 4.2 mg/ml (referred to hereafter as 3.5S). The fragment was apparently unstable in the presence of papain since after 8 hr of digestion there was no longer any detectable material except in the small molecular weight region of the column.

Of interest was the observation that partial reduction of shark $19S_{NAT}$ or $7S_{NAT}$ resulted in molecules ($19S_{RED}$ and $7S_{RED}$) which were identical with $7S_{NAT}$ with respect to sedimentation properties, molecular weights, and antigenic composition. Yet under conditions of proteolysis comparable to those used for $7S_{NAT}$, these molecules had somehow been altered and made susceptible to more extensive degradation. As a control experiment, rabbit IgG was partially reduced and digested with papain for 8 hr. Most of the protein had an $s_{20,w}$ of 3.4 S (7.2 mg/ml). This was the same sedimentation coefficient obtained for rabbit IgG which had not been reduced and alkylated prior to papain digestion.

Trypsin Digestion of Partially Reduced and Alkylated Lemon Shark Immunoglobulins. The results with papain fragmentation of partially reduced lemon shark immunoglobulins suggested that it would be worthwhile to study tryptic hydrolysis of this same material. Digestions were performed as described for tryptic fragmentation of $7S_{NAT}$ and the products were examined by sedimentation velocity. Results with $19S_{RED}$ were similar, so only the case of $7S_{RED}$ will be considered here. The calculated sedimentation coefficient after 30 min of digestion was 6S for the major peak, with a slower sedimenting shoulder being obvious. After 8 hr, the major component had an $s_{20,w}$ of 3.6 S at a concentration of 3.7 mg/ml (considered equivalent to the 3.5S fragments discussed previously) with a small amount (<5%) of 10S material, presumably nonspecific aggregate (which has not been further characterized), also seen. The 3.5S fragment was quite stable in the presence of trypsin, and even after 24 hr, its yield was only 10–15% less than after 8 hr of digestion.

The molecular weight (Table I) of this fragment was calculated by equilibrium centrifugation and was found to be ~50,000. A sample (0.2 mg) of 3.5S material was labeled with ^{125}I and extensively reduced and alkylated along with 30 mg of rabbit IgG in the presence of 7 M guanidine·HCl. The mixture of polypeptide chains was then chromatographed on the calibrated Sephadex G-200 column equilibrated in 5 M guanidine·HCl to determine molecular weights of the remaining H and L chains of the shark proteolytic fragment. Most (67%) of the radioactivity eluted in the same volume as rabbit L chain (mol wt ~22,500). It appeared from this column profile that the μ chain had been further degraded by trypsin until it now approached the size of a light chain. Again, cleavage of a presumably limited number of disulfide bonds altered the structure of the molecule so that it was at least somewhat more susceptible to tryptic proteolysis but still not as susceptible as to papain.

Immunoelectrophoresis of this 3.5S molecule indicated that it had not changed in mobility from the intact $7S_{NAT}$ or any of the other fragments produced during this study. Ouchterlony analysis of $4.5S_{RED}$ and $3.5S_{Try}$ was performed utilizing three different rabbit antisera to $7S_{NAT}$. Only one of these showed the $3.5S_{Try}$ molecule to be antigenically deficient to $4.5S_{RED}$. Neither the anti-H-chain nor anti-L-chain antisera showed this difference. It is therefore rather tenuous to say at this point whether the enzyme was further fragmenting H chains or possibly both H and L chains were being digested.

Tryptic Hydrolysis of $4.5S_{RED}$. In view of the finding that the "core" fragment resulting from reduction of the $6S_{Pap}$ obtained from unreduced $7S_{NAT}$ was a 4.5S piece, and that the fragment resulting from digestion of $7S_{RED}$ or $19S_{RED}$ was a 3.5S piece, it was decided to subject reduced 4.5S fragments to further tryptic hydrolysis. A sample (7 mg) of $4.5S_{RED}$ was prepared as described ($7S_{NAT} \xrightarrow{\text{papain or trypsin}} 6S \xrightarrow[SH]{} 4.5S_{RED}$) and digested with trypsin in the pH-Stat for 8 hr under the conditions previously stated for kinetic analysis. The calculated $s_{20,w}$ of this material was ~3.5 S. This result clearly indicated that partial reduction of the shark immunoglobulin molecule resulted in a change in configuration of that molecule making it more susceptible to proteolytic attack, yet there remained a resistant "core" particle of 3.5S material.

Hexose Content of Lemon Shark Immunoglobulin and Its Fragments. Hexose determinations were performed on proteolytic fragments and isolated polypeptide chains derived from lemon shark 7S IgM. Table II presents the results of these experiments. It can be seen that both 6S and its reductive cleavage product ($4.5S_{RED}$) have comparable hexose contents, which are lower than the hexose level of the intact 7S molecule. Further enzymatic degradation of the H chain lowers the hexose content to a level comparable to L chain alone, suggesting that the "Fd" fragment has little or no hexose associated with it.

Kinetic Studies of Papain and Trypsin Digestion of Lemon Shark Immunoglobulins. One of the major problems in attempting to elucidate where the intersubunit disulfide bonds are located in an IgM molecule is the fact that the Fc region of this molecule is very difficult to isolate. Since the major product of either papain or tryptic digestion of $7S_{NAT}$ was a 6S fragment, and it was apparent that there was a

TABLE II: Hexose Content of Lemon Shark 7S IgM and Its Proteolytic Fragments.

Protein	Hexose Content (%)
$7S_{NAT}$	4.4
$6S_{Pap}$	3.5
$4.5S_{RED}$	3.7
3.5S	1.3
$7S_{NAT}$ H chain	4.0
$7S_{NAT}$ L chain	1.0

difference in the time required to ultimately reach this size, it was considered of interest to follow the kinetics of fragmentation.

Digestions were performed in a recording pH-Stat and a pK of 7.85 for newly liberated α-amino groups (Cohen and Edsall, 1943) was assumed. The data obtained from the pH-Stat has been converted from the actual experimental data (*i.e.*, NaOH added plotted against time) into the number of peptide bonds broken per 180,000 dalton unit and plotted against time (Figure 4). It can be seen that 50% of the bonds which would ultimately be broken by papain had been hydrolyzed after approximately 30 min of digestion. On the other hand, it took more than 90 min for trypsin to hydrolyze 50% of the bonds it would eventually split. It must be noted that 200 min represents essentially the culmination of the reaction with papain (as evidenced by no additional hydrolysis after addition of extra enzyme) and the tryptic digestion would stop within the next 30 min. Thus, these data agreed with that presented earlier with respect to different rates of formation of the 6S product. The number of peptide bonds hydrolyzed per 7S Ig molecule (~80) is consistent with the production of dialyzable peptides from the cleaved segment (~70,000 daltons) and the total digestion of an undetermined, but small amount of the starting immunoglobulin preparation.

Treatment of the kinetic data from the pH-Stat in an attempt to delineate a possible two-step reaction in a fashion similar to that used for myosin (Mihalyi and Harrington, 1959) was inconclusive in spite of repeated experiments. It was quite probable that very shortly after addition of enzyme to $7S_{NAT}$, a multitude of distinct reactions were going on simultaneously. It has thus far proved experimentally impossible, by such means as digesting at different temperatures (4°, 22°) and pH values (7.4, 8.0) to alter these reactions in order to delineate a "fast" and "slow" sequence of proteolytic events.

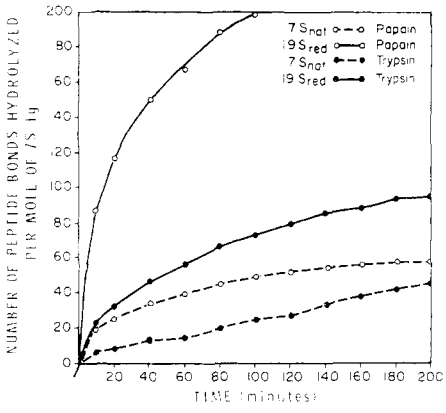

FIGURE 4: Plot of the number of peptide bonds broken *vs.* time using both papain and trypsin to digest lemon shark immunoglobulins.

Both $19S_{RED}$ and $7S_{RED}$, digested under these same conditions, showed more extensive hydrolysis than $7S_{NAT}$. The data for digestion of $19S_{RED}$ by papain have also been plotted in Figure 4. Similar results were obtained with $7S_{RED}$. This reaction was only followed for 100 min, and clearly the reaction had not yet stopped at this point. But it was clear that hydrolysis was considerably more extensive than during papain digestion of $7S_{NAT}$ which supported the observation made previously that papain digestion of $19S_{RED}$ or $7S_{RED}$ resulted eventually (by 8 hr) in its conversion into low molecular weight peptides.

Discussion

The work reported here is part of a study aimed eventually at understanding the relationship between the two molecular forms of shark IgM and the relationship between these molecules and the various forms of IgM (Rothfield *et al.*, 1965; Stobo and Tomasi, 1967; Perchalski *et al.*, 1968; Hunter *et al.*, 1968) in higher vertebrates. Knowledge of the gross architecture of these proteins is important from a comparative viewpoint and perhaps eventually will result in a model for pentameric IgM synthesis in vertebrates in general.

Considerable work has been done during the past few years with both tryptic and papain digestion of human Waldenström IgM proteins, and it would seem appropriate here to discuss the major findings with these proteins.

The bulk of the data to be discussed below is summarized in Figure 5. It should also be mentioned here that although IgM proteolytic fragments are identified by slightly different sedimentation coefficients in various laboratories, fragments of 6S and 6.1S are considered comparable as are those 4.3S to 4.5S and likewise those ranging from 3.2S to 3.7S.

Miller and Metzger (Miller and Metzger, 1966) digested a Waldenström 19S protein with trypsin and obtained a 6.1S fragment which was designated $F(ab\mu')_2$ and a 3.7S fragment designated Fabμ. Partial reduction of the 6.1S fragment resulted in its splitting into two apparently identical 4.4S fragments; these latter fragments could then be further digested to 3.7S material. Trypsinolysis of the reductive 7S subunit resulted in the liberation of a 4.3S fragment which after longer proteolysis was degraded to 3.7S material; this latter fragment was very similar to the previously mentioned Fabμ. No Fcμ fragments were obtained in this study; this portion of the molecule was apparently degraded to small peptides by proteolysis. Onoue *et al.* (Onoue *et al.*, 1966, 1968a), working with another Waldenström 19S protein, found that papain digestion (performed with preactivated enzyme in the absence of reducing agents) resulted in the liberation of 3.2S fragments corresponding to Miller and Metzger's 3.7S (Fabμ) fragments. These latter studies did however, result in the recovery of an Fcμ-like fragment (Onoue *et al.*, 1968a). Digestion of either the 19S molecule in the presence of reducing agent or of the reductive 7S subunit, both resulted in the recovery of 3.2S Fabμ fragments although no Fcμ was obtained under these conditions. Additional workers (Mihaesco and Seligmann, 1967; Inman and Hazen, 1968; Goodman and Inman, 1969; Chen *et al.*, 1969; Beale, 1969; Suzuki, 1969) have studied other Waldenström proteins, and their results tend to support the above

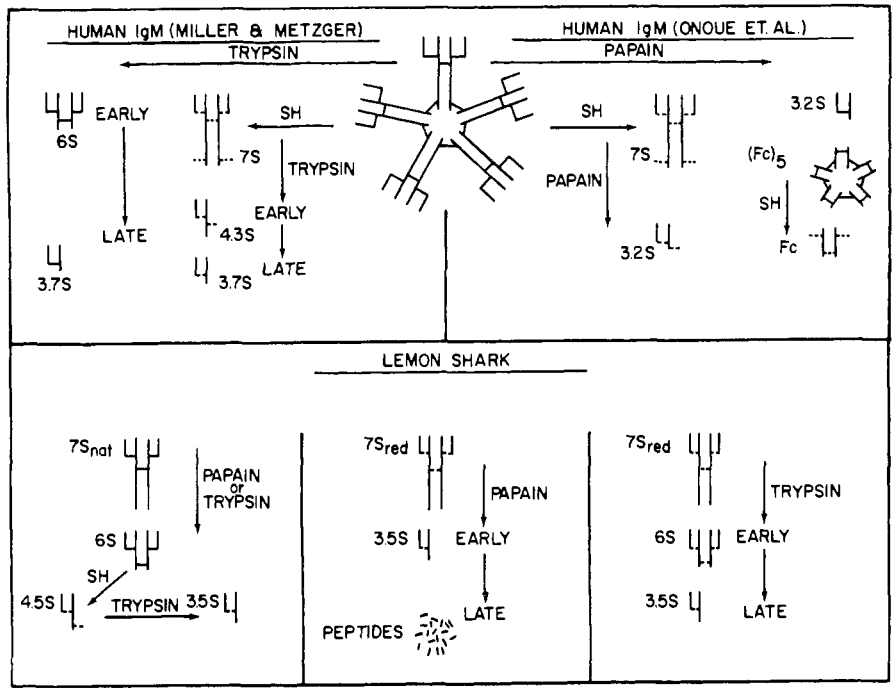

FIGURE 5: Schematic representation of results of papain and tryptic digestion of IgM depicted for studies by Miller and Metzger (1966), Onoue *et al.* (1966, 1968a,b), and the findings in lemon sharks. Intact interchain disulfide bonds (|—|), reduced interchain disulfide bonds (|----|). Format taken from Miller and Metzger (1966).

results including the "elusiveness" of Fcμ. It should also be mentioned that the Fabμ fragments were so designated based solely upon structural anlogy with the IgG molecule and not upon the fact that this region of the IgM molecule contained the antibody-combining site. However, Ashman and Metzger (Ashman and Metzger, 1969) working with a Waldenström protein with anti-DNP activity and Onoue *et al.* (Onoue *et al.*, 1968b) with rabbit IgM antibody to a hapten were able to demonstrate that Fabμ does in fact contain one antibody-combining site.

With the above background discussion relative to the organizational structure of human and rabbit IgM, as elucidated by proteolysis, it would now be appropriate to focus on the lemon shark immunoglobulins in this context. First of all, it should be mentioned that other workers have claimed that elasmobranch immunoglobulins were resistant to proteolysis by papain (Marchalonis and Edelman, 1968) since they noted no electrophoretic changes after digestion. Since the various fragments obtained in the study reported here had immunoelectrophoretic mobilities comparable to that of the undigested native protein, it seems reasonable to suggest that proteolytic fragments would not be differentiated from undigested material by such techniques.

Digestion of the native lemon shark 7S immunoglobulin with trypsin or papain resulted in the liberation of a 6S fragment (mol wt ~110,000) and some small dialyzable peptides. Mild reduction of the 6S fragment resulted in its splitting into two 4.5S (mol wt ~60,000) fragments. Antigenic analysis showed the 6S and 4.5S fragments to be identical and while containing all the L-chain determinants, they appeared to be missing some H-chain determinants present in the native molecule. The missing H-chain determinants presumably were represented by the missing 27,000 daltons (the small dialyzable peptides). Further digestion of the 4.5S fragment with trypsin resulted in its degradation to a "stable" 3.5S fragment. Carbohydrate determinations showed that ~20% of the hexose of $7S_{NAT}$ was lost in converting the native molecule into 6S or 4.5S fragments and 80% of the total hexose is removed if one further digests to 3.5S. The similarity of the shark fragments to those obtained by other workers with human IgM is also evident in Figure 5. However, since the studies reported here were done with "normal" lemon shark immunoglobulin and no antibody activity was studied, any designation of the shark fragments relative to human IgM fragments must be considered provisional. Preliminary results have indicated that the nurse shark 7S immunoglobulin behaves in a similar fashion as the lemon shark 7S molecule to trypsinolysis, *i.e.*, 7S $\xrightarrow{\text{trypsin}}$ 6S $\xrightarrow{\text{SH}}$ 4.5S. Thus, the finding that the 6S fragment obtained from specifically purified nurse shark 7S anti-*Salmonella typhimurium* O antigen agglutinated *Salmonella typhimurium* cells as efficiently as the native 7S antibody, indicates each to contain antibody-combining sites (presumably two). The 4.5S fragment obtained by reduction from this nurse shark 6S antibody fragment no longer agglutinated bacterial cells, but still bound to the antigen as well as the 6S fragment (measured by binding of ^{125}I-labeled fragments to cells), (D. G. Klapper and L. W. Clem, unpublished observations). Thus, it appears that the lemon shark 6S fragment most likely also contains combining sites and can be considered as being analogous to F(abμ')$_2$. These data also indicate the 4.5S fragment to be comparable to Fabμ; actually the shark "core" 3.5S fragment should more appropriately be considered as Fabμ.

The gross structure (molecular weight and numbers of H and L chains) of the shark native 7S immunoglobulin and the partially reduced 7S proteins ($7S_{RED}$ and $19S_{RED}$) are quite similar. However, the splitting of a presumably limited number of disulfide bonds has changed their susceptibility to proteolysis. Papain digests the reduced molecules to a 3.5S fragment(s) which is quickly digested further to small dialyz-

able peptides. Trypsinolysis, on the other hand, results in the production of a 6S fragment followed shortly thereafter by the appearance of 3.5S fragments. The important point here appears to be that each of these proteins contains a "core" of a 3.5S material (representing Fabμ) which is relatively resistant to proteolysis. Thus, since the primary function of antibody is to bind antigen, and since this property is dependent upon conformation of the antibody site, it is reasonable to suppose that the Fab of shark and man is protected from proteolysis by the amino acid sequence and the resultant three-dimensional structure in the "area" of the active site.

An unanswered question regarding the proteolysis of shark immunoglobulins is whether or not an Fcμ fragment is present and if so does it have similar biologic functions to the Fc fragment from the immunoglobulins of higher animals, *i.e.*, complement fixation. Presumably the small dialyzable peptides liberated during proteolysis represent the Fcμ structural region. Future studies aimed at isolating this portion of the shark immunoglobulins must consider different approaches such as sodium borohydride cleavage (Yakulis and Schmale, 1968) or high-temperature proteolysis (Plaut and Tomasi, 1970). Preliminary data suggest that neither of these techniques, when applied to the shark molecule, results in the production of intact Fcμ fragments.

In conclusion, it appears that although there was "evolution" of multiple immunoglobulin classes from sharks to man, there does not appear to have occurred significant changes in the immunoglobulin molecules, as evidenced by enzyme susceptibilities in the same general regions. One might, however, speculate from the difference in papain susceptibility of partially reduced shark IgM, on the one hand, and partially reduced mammalian IgM and IgG, on the other, that the phylogenetically ancient shark has an immunoglobulin structure less well stabilized by noncovalent bonds after partial reduction and that evolution of these essential molecules has resulted in a more stable "core" fragment.

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