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Studies on the Chymotrypsin C and Papain Fragments of Human Immunoglobulin M*

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ABSTRACT: Human Waldenstrom macroglobulins and their reductive subunits were subjected to proteolysis by chymotrypsin C (isolated from porcine pancreas) and papain. Chymotrypsin C cleaved immunoglobulin M into three fragments which had sedimentation coefficients of CI = 16.6 S, CII = 6.6 S, and CIII = 3.8 S. Antigenic analysis showed that CIII with the molecular weight of 40,000 corresponds to an Fabμ fragment. CII with the molecular weight of 135,000 is a dimer of CIII plus an additional fragment of 55,000. CI is a mixture which can be resolved by equilibrium ultracentrifugal technique into two major components with weight-average molecular weights of 773,000 and 606,000, and is composed of immunoglobulin M molecules from which one or more F(ab')$_2\mu$ fragments have been hydrolyzed. The calculation of the yield of Fabμ from proteolysis of the reductive subunit demonstrated the presence of 2 Fabμ units in the 7S subunits and 10 in the parent 19S immunoglobulin M. Short-term digestion of immunoglobulin M by papain produced three fragments which were separable on Sephadex gel filtration and had sedimentation coefficients of PI = 18.4 S, PII = 7.0 S, and PIII = 3.7 S. PIII contains two components corresponding to Fabμ and Fcμ fragments. After longer periods of incubation with papain, PIII (Fabμ fragments) with the molecular weight of 37,000 are the only immunologically reactive fragments detectable and the remainder of the molecule is degraded into peptides. It is believed that in the presence of 0.01 M cysteine immunoglobulin M is reduced to the reductive subunit of immunoglobulin M (PII) and then the 7S subunit is degraded into PIII by papain. PI is most likely the unreduced and undigested immunoglobulin M. Quantitative complement fixation techniques have been used to study the immunological relationships between the proteolytic fragments and to localize the antigenic determinants of various antisera including those responsible for the individual (idiotypic) specificity.

Nearly one-third of the total hexose found in the reductive subunit of immunoglobulin is bound to an F(ab')$_2\mu$ fragment. Hence, over two-thirds of the carbohydrate resides in the C-terminal region beyond the chymotrypsin C cleavage. The results of these studies are consistent with a circular pentameric model of immunoglobulin M containing 10 Fabμ units.

Miller and Metzger (1965) proposed that 19S IgM[1] molecules consist of five 7S subunits (IgMsb) which can be liberated by reduction and alkylation. This model is substantiated by quantitative studies involving molecular weights and yields of subunits (Miller and Metzger, 1965) and numbers of interchain disulfide bonds (Miller and Metzger, 1965) and is supported by recent electron microscopic evidence presented by Svehag *et al.* (Svehag *et al.*, 1967; Chesebro *et al.*, 1968).

Enzymatic degradative studies by Miller and Metzger (1966) using trypsin showed that tryptic hydrolysis of IgM yielded

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[1] The abbreviations used are in accordance with the recommendations of the World Health Organization. The one not listed in *Bull. World Health Organ. 30*, 447 (1964) is: IgMsb, the reductive subunit of IgM.

both Fabμ and F(ab')$_2\mu$ fragments. F(ab')$_2\mu$ fragment appeared to be a dimer of the Fabμ fragments (although somewhat larger) linked by a single disulfide bond *via* their H chains. Thus their studies suggested that each 7S reductive subunit (IgMsb) is composed of a pair each of H and L polypeptide chains linked by disulfide bonds. Furthermore, the intersubunit disulfide bonds have been localized in the Fcμ portion of the IgM molecule by the studies of Mihaesco and Seligmann (1968b) and Onoue *et al.* (1968b) who succeeded in the isolation of, using limited proteolysis with papain, a high molecular weight Fcμ fragment containing polymers of smaller Fcμ-like fragments.

A 20 polypeptide chain model of IgM with 10 H (heavy) and 10 L (light) chains would therefore be expected to possess 10 antibody combining sites as inferred from the molecular model of IgG. However, some immunological studies show that the valence of the 19S molecule is 5 rather than 10 (Franklin *et al.*, 1957; Onoue *et al.*, 1965; Metzger, 1967; Schrohenloher and Barry, 1968) and upon reduction and alkylation univalent 7S subunits are produced. It is interesting to note three recent reports: the first suggesting the number of the binding sites of human IgM to be 10/19S molecule (Merler *et al.*, 1968), the second showing that the 7S subunit of cold agglutinins may have bivalent antibody activity (Cooper, 1967), and the third demonstrating that each of the 7S subunits of the IgM molecule has one strong and one weak combining site (Onoue *et al.*, 1968b). Perhaps pertinent to the question of IgM valency is a different chain model of IgM with 10 H and 15 L chains recently proposed by Suzuki and Deutsch (1967). They suggest that the reduced subunit of IgM may have 3 rather than 2 L chains. Following alkylation, 2 of the L chains are released while 1 remains firmly attached to the H chains to give the reduced-alkylated subunit. This interesting hypothesis awaits verification in other laboratories.

In view of these partially conflicting reports, further structural studies of IgM by means of enzymatic cleavage seem desirable. Admittedly most Waldenstrom IgM do not have demonstrable antibody activity although well-described exceptions have been reported (Metzger, 1967; Stone and Metzger, 1967). However, the available evidence suggests that they may be regarded as individual species of the normal IgM population, and therefore further studies on these proteins are relevant to the problem of the structure and valency of the IgM antibody.

Gjessing and Hartnett (1962) reported the isolation and crystallization of an esteroproteolytic enzyme from porcine pancreas. Chymotrypsin C (Folk and Schermer, 1965; Folk and Cole, 1965) has likewise been isolated from porcine pancreas and its specificity toward various polypeptides has been studied. It is apparent from the data presented that chymotrypsin C is very similar to the esteroproteolytic enzyme of Gjessing and Hartnett (1962). However, the molecular weight determinations suggest that the enzyme isolated by Folk and Schermer (1965) is of a smaller size perhaps as a result of partial autolysis during isolation (E. C. Gjessing, 1968, personal communications).

We have used the Gjessing enzyme (hereafter called chymotrypsin C), whose proteolytic activity on γ-globulin resembles although is not identical with that of trypsin, to subject Waldenstrom's macroglobulins and their reductive subunits to proteolytic cleavage. The cleavage products have been separated and these proteolytic fragments were compared with each other immunologically and with the products of papain digestion of IgM.

Materials

IgM Immunoglobulin. The majority of the work reported here was performed on a macroglobulin preparation obtained from an individual patient. However, to be certain that the results were not unique to this protein, four other macroglobulin preparations were also studied in a similar manner with essentially identical results.

The frozen plasma of a patient with Waldenstrom's macroglobulinemia (DiS; plasmapheresis) was thawed and bovine thrombin and $1/_{50}$ in volume of 40% $CaCl_2$ were added. The fibrin clot was removed by centrifugation. Euglobulin was prepared by diluting the serum $1/_{15}$ with deionized water. The precipitated protein after centrifugation was dissolved in a minimal volume of 0.15 M NaCl and the solution was applied to a Sephadex G-200 column and eluted with isotonic saline. Only the eluates which showed a single IgM line in Ouchterlony gel diffusion with a rabbit antiserum prepared against the euglobulin of the same patient were pooled and concentrated. These eluates also showed a single precipitating system when tested against rabbit anti-whole human serum and gave no reaction with anti-IgG or IgA antiserum; 19S IgM was separated from dimers and trimers of IgM by agar gel chromatography, using Sagarose-6 purchased from Seravac Laboratories.

Proteolytic Enzymes. Chymotrypsin C (Gjessing and Hartnett, 1962) which had been isolated from porcine pancreas by fractionation on DEAE-cellulose and recrystallized three times was kindly provided by Dr. Gjessing of the University of Vermont. Papain was purchased from Sigma as a 27.5-mg/ml suspension in 0.05 M sodium acetate (pH 4.5).

Antisera. Antisera were prepared by immunizing rabbits with subcutaneous and intraperitoneal injection of 3 mg each of antigens in complete Freund's adjuvant at intervals of 1–2 weeks over a period of 3–4 months. Antiserum 18 was produced by immunization with the euglobulin of patient DiS (λ type) and contained antibodies specific for IgM. This antiserum was made μ-chain specific by absorbing it with human cord serum. Antiserum 1c was prepared by injecting pellets obtained from normal human serum in a preparative Model L ultracentrifuge. Antiserum 71 is directed against the heavy (μ) chains isolated from the IgM of patient Reg. as described in the Method section. Antiserum 74 was prepared by immunization with the monoclonal IgM of patient Mur (κ type). Antiserum 24 was made by injecting the Fab fragment of human IgG (Lederle Fraction II) which was isolated from the papain digest of the latter by starch block electrophoresis. This antiserum contained antibodies directed against both κ- and λ-type L chains.

Methods

Enzyme Digestion. Chymotrypsin C digestion was performed at 37° in 0.1 M sodium phosphate buffer (pH 8.0) using 1–2% of enzyme by weight. The digest was inactivated with a 3 molar excess of DFP. Papain hydrolysis was carried out following the method of Porter (1959). The reaction mixture was then inactivated with 0.01 M *p*-mercuribenzoate (sodium) or 0.01 M iodoacetamide. In some experiments with papain, shorter periods (1.5 hr) of digestion were used.

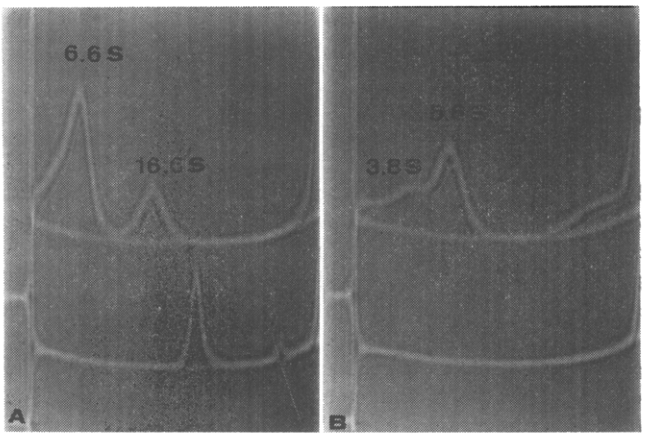

FIGURE 1: Sedimentation patterns of the 24-hr chymotrypsin C digest of IgM (top) and IgM control (bottom). Wedge window (top) at 29.3 mg/ml and bottom pattern at 4.4 mg/ml in 0.1 M sodium phosphate buffer (pH 8.0). The schlieren photograph on the left (A) was taken 32 min after attaining the speed of 52,640 rpm and the picture on the right (B) after 64 min.

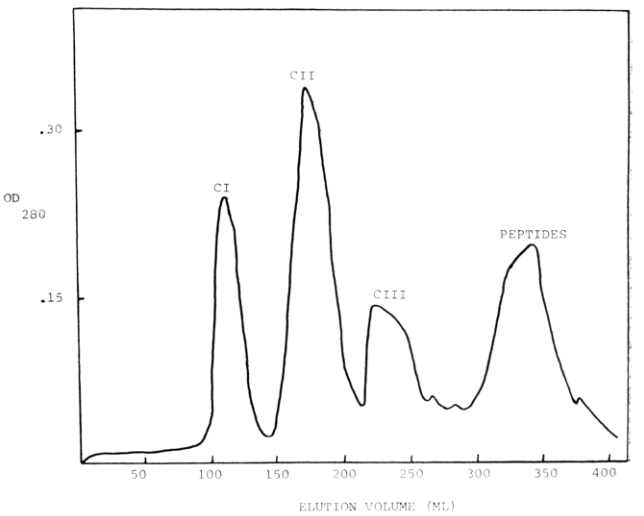

FIGURE 2: Elution profile of a 24-hr chymotrypsin C digest of IgM from a Sephadex G-200 column (2.3 × 97 cm) equilibrated with 0.15 M NaCl.

Reductive Cleavage. To IgM in 0.1 M sodium phosphate buffer (pH 8.0) an equal volume of 0.02 M dithiothreitol in the same buffer was added and allowed to stand at 25° for 2 hr with occasional mixing. Following reduction the reaction mixture was chilled in an ice bath and treated with a 10% excess of iodoacetamide for 1 hr at 0° and subsequently dialyzed against a large volume of 0.15 M NaCl in the cold. H and L chains of IgM were separated by gel filtration of the reduced and alkylated IgM on Sephadex G-100 in 1 N propionic acid or 4 M guanidine hydrochloride. Iodoacetamide (K & K Laboratories) was recrystallized from hot water and air dried (mp 94–95°). Guanidine hydrochloride was prepared by the acidification of guanidine carbonate (Eastman) and recrystallized from an ethanol–benzene mixture. The proteolytic fragments of IgM were reduced with 0.001 M dithiothreitol in 0.1 M sodium phosphate buffer for 1 hr at pH 8.0 and 25°. The liberated sulfhydryl groups were blocked by dialysis against 0.02 M iodoacetamide in pH 7.5 phosphate buffer (ionic strength 0.1) in the cold for 5 hr, and finally the sample was dialyzed overnight against a large volume of 0.15 M NaCl.

Ultracentrifugation. Analytical ultracentrifugation was carried out in a Spinco Model E ultracentrifuge at 52,640 rpm and 20°. Infinite dilution values were obtained from plots of s *vs.* concentrations. The concentrations of IgM and IgMsb were determined by reading the optical density at 280 mμ in a Zeiss PMQ II spectrophotometer. $E_{280}^{1\%}$ 11.8 was used (Miller and Metzger, 1965). The concentrations of the proteolytic fragments were measured as follows. Peak areas were determined by projection of enlarged schlieren patterns on graph paper, and relative areas were determined by counting squares. The corresponding protein concentrations were calculated from the equation given by Schachman (1957), using a value of 1.87×10^{-3} for the specific refractive increment (Doty and Geiduschek, 1953) assuming that the fragment has the same increment as human γG-globulin.

The molecular weights of IgM and its fragments were determined by the meniscus depletion method of Yphantis (1964), employing the interference or ultraviolet absorption optics. The partial specific volume, $\bar{v}$, of 0.717 was calculated from the amino acid composition data of human IgM given by Heimburger *et al.* (1964) and the carbohydrate data of human IgM by Miller and Metzger (1965). The values of 0.62 (for hexoses and hexosamine) and 0.59 (for sialic acid) were assumed (Bezkorovainy and Doherty, 1962).

For the molecular weights of the IgM fragments poor in carbohydrate content, their partial specific volumes were calculated by assuming that the decrease in per cent content of hexosamine and sialic acid are proportional to that of hexose. The values of $\bar{v}$ of 0.724 for CII and 0.725 for CIII and PIII were obtained. The sedimentation equilibrium runs were carried out in a multichannel cell at three protein concentrations ranging from 0.1 to 1.0 mg per ml. The calculated molecular weight was the average of the results obtained at the various protein concentrations.

Immunology. Immunoelectrophoresis was performed according to the micromethod of Scheidegger (1955) with 2% agar agar in 0.05 M barbital buffer (pH 8.2). Ouchterlony double-diffusion analyses were carried out in 1% Bacto agar in 0.15 M NaCl and 0.1% NaN_3. Quantitative complement fixation experiments were done following the procedure of Mayer *et al.* (1948).

Carbohydrate Determination. Hexose content was determined by the orcinol–H_2SO_4 method of Rosevear and Smith (1961) which is a slight modification of the one described by Winzler (1955). Standard curves were constructed using a mannose–galactose–fucose standard consisting of 24, 12, and 5 mg % for each sugar over the range of 0–450 μg. Orcinol (Fisher reagent grade) was recrystallized from benzene. The concentrations of IgM and IgMsb were determined from their extinction coefficient ($E_{280}^{1\%}$ 11.8). The protein concentrations of IgM fragments were measured by the Biuret reaction (Kabat and Mayer, 1961).

Disc Electrophoresis. Electrophoresis was performed according to the method of Ornstein and Davis (Ornstein, 1964; Davis, 1964). The bottom gel was 5% instead of the usual 7.5% to accommodate larger protein molecules.

Results

IgM Immunoglobulin. The ultracentrifugation pattern of the

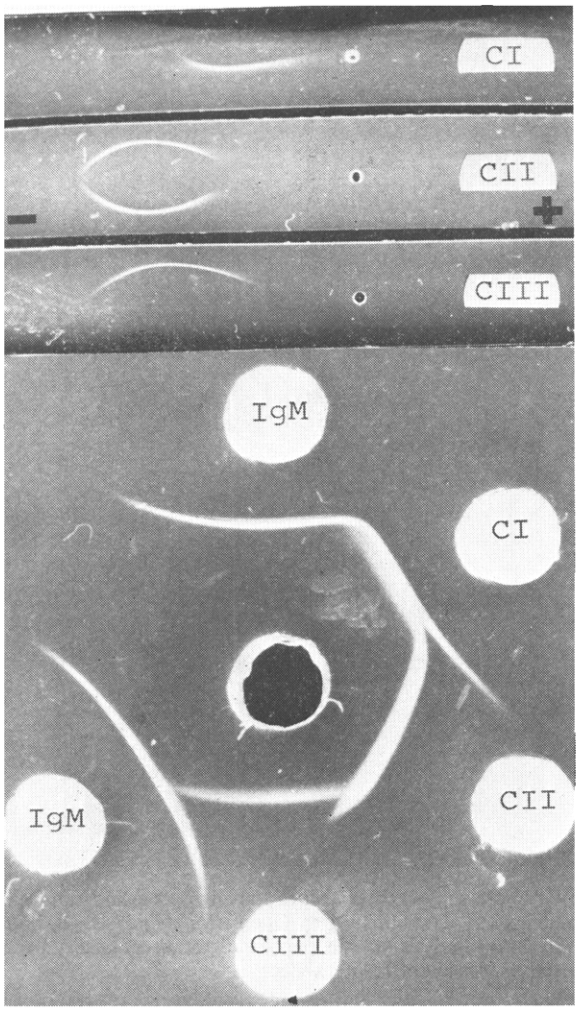

FIGURE 3: Immunoelectrophoretic and double gel diffusion analyses of the chymotrypsin C digestion products of IgM. (A, top) Immunoelectrophoresis with rabbit 18 anti-IgM serum; (B, bottom) double gel diffusion with antiserum 18.

euglobulin preparation showed a major component with a sedimentation rate of 16.4 S and smaller amounts of 22.4S and 28.9S polymers at a protein concentration of 10.8 mg/ml. The preparation was found by immunological criter a to be contaminated with a small quantity of IgG which was removed by passage through a Sephadex G-200 column. For enzyme hydrolysis and reductive cleavage, the IgM preparations containing h gher molecular weight components were used directly. For molecular weight determinations, homogeneous 19S IgM was isolated by gel filtration through Sagarose-6.

Chymotrypsin C Digestion of IgM. IgM when incubated with 2% weight (with respect to IgM) of chymotrypsin C for 24 hr and examined in the analytical ultracentrifuge gave the pattern seen in Figure 1. The three peaks which are visible in the sedimentation pattern represent proteins having sedimentation coefficients of 16.6, 6.6, and 3.8 S after isolation by gel filtration. Upon incubation of IgM with chymotrypsin C for 48 hr, the 6.6S peak disappears in the schlieren pattern and there remain only proteins with sedimentation coefficients of approximately 15 and 3.8 S.

Resolution of the 24-hr digest of IgM was accomplished by chromatography on a Sephadex G-200 column equilibrated

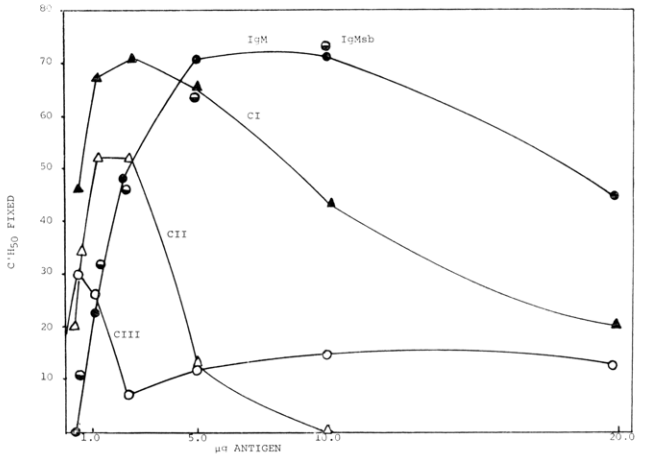

FIGURE 4: Fixation of complement by IgM, IgMsb, and the chymotrypsin C fragments of IgM in the presence of antiserum 18 (anti-IgM) diluted $^1/_{400}$. Symbols for the curves are as follows: IgM (●), IgMsb (◒), CI (▲), CII (△), and CIII (○).

with 0.15 M NaCl (saturated with toluene). The elution pattern is shown in Figure 2. Four well-resolved peaks were obtained. These peaks, starting with the void volume, were designated as CI (16.6 S), CII (6.6 S), CIII (3.8 S), and peptides plus enzyme. The areas under each peak amounted to 17% for CI, 36% for CII, 15% for CIII, and 33% for peptides plus enzyme. The pooled fractions from peak CII and CIII were shown to be homogeneous by the criteria of immunoelectrophoresis and disc electrophoresis. Immunoelectrophoretic analyses (Figure 3A) revealed CII and CIII as electrophoretically slower moving materials than CI and IgM. On disc electrophoresis, CII and CIII migrated as slower moving single bands while CI and IgM did not penetrate the polyacrylamide gel in the unreduced state.

Using anti-IgM antiserum 18 (absorbed with cord serum), CI is antigenically equivalent to the native IgM. However, CII is antigenetically deficient compared with CI, whereas CIII is deficient compared with both CI and CII (Figure 3B). Using an L-chain-specific antiserum (antiserum 24) all of these protein molecules exhibited immunological identity.

Results of quantitative complement fixation (Figure 4) parallels those of the Ouchterlony analysis. However, it is interesting to note that CI seems to be more efficient than the whole IgM, in that it reaches the maximum in complement fixed more rapidly. Another point of interest is that the reduced and alkylated IgMsb showed a broad curve of high maximum similar to that of nat ve IgM. The second diffuse plateau in the CIII curve is probably due to a small amount of impurities. It should be pointed out that the C′-fixation curves of the patient's (DiS) whole serum and isolated IgM were indistinguishable with respect to antiserum 18.

Chymotrypsin C Digestion of IgMsb. IgM was reduced in the presence of dithiothreitol and alkylated with iodoacetamide as described in the Methods section. As noted previously by Suzuki and Deutsch (1966), the 7S IgM subunit gave similar slow arcs with both rabbit 18 (anti-IgM) and rabbit 24 (anti-L-chain) sera. However, when reacted with another anti-μ-chain antiserum (rabbit 71), a second faster arc of specific precipitation is seen. The fast arc which does not contain L-chain determinants probably represents free H chains.

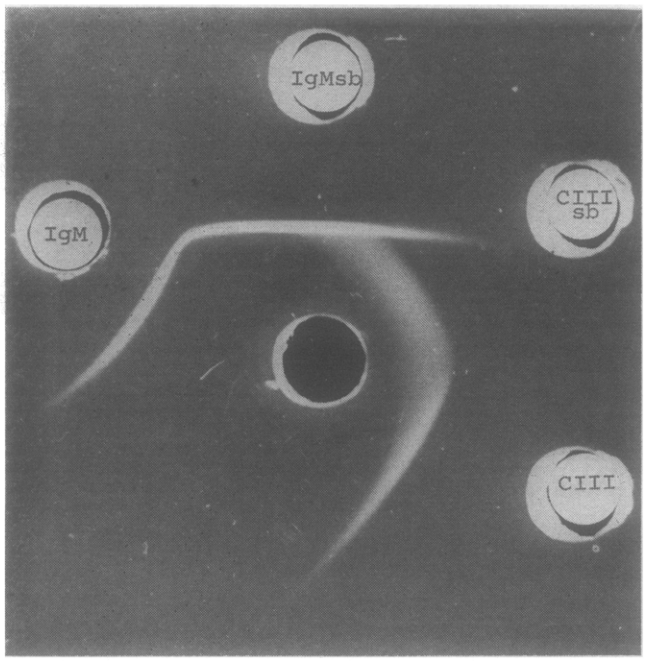

FIGURE 5: Comparison of IgM, IgMsb, and chymotrypsin C fragments derived from IgM and IgMsb by Ouchterlony analysis using antiserum 18 (anti-IgM) in the center well.

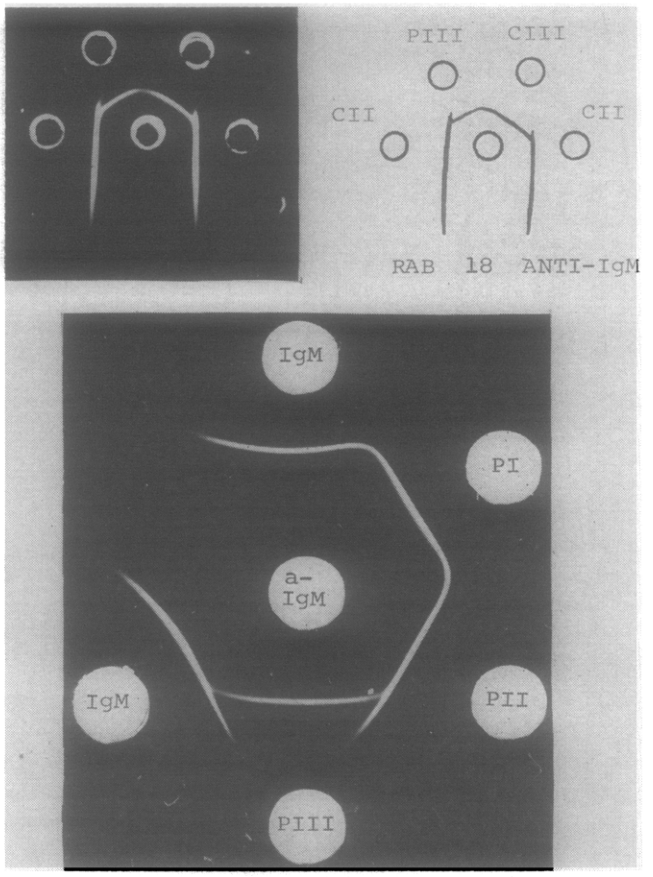

FIGURE 6: (A, top) Comparison of the chymotrypsin C and papain fragments derived from IgM by Ouchterlony analysis. (B, bottom) Ouchterlony analysis of the papain digestion products of IgM. Antiserum 18 is in both center wells.

IgMsb was treated with 1% by weight (in relation to IgMsb) of chymotrypsin C for 9–18 hr at 37° in 0.1 M sodium phosphate buffer (pH 8.0). Digestion was terminated by adding DFP and the digest was scanned in the analytical ultracentrifuge. A single peak was present with the sedimentation coefficient of 3.6 S after isolation by gel filtration on Sephadex G-100. The peak which corresponds immunologically to CIII (obtained from the whole molecule) is designated CIIIsb (Figure 5). Immunoelectrophoresis shows only a single slow arc paralleling in mobility that of CIII. No Fc-like fragment has been observed on immunoelectrophoresis with nine different anti-μ-chain sera. An electrophoretically fast fragment could not be produced from IgMsb with this enzyme even with an incubation time as short as 30 min.

The yield of CIIIsb was determined as follows. To 5.5 mg of IgMsb (reduced with 0.01M dithiothreitol but not alkylated) in 1 ml of 0.1 M sodium phosphate buffer (pH 8.0) was added 1% by weight of chymotrypsin C and the reaction mixture was incubated at 37° for 18 hr. The digest was dialyzed against a large volume of 0.1 M sodium phosphate buffer (pH 8.0) overnight to remove the dialyzable material. There was no significant change in the volume of the protein solution within the dialysis bag. The resulting CIIIsb was viewed with the reduced IgMsb (the original undigested IgMsb) in a double-sector cell in an analytical ultracentrifuge. Peak areas were estimated by projection of enlarged schlieren patterns on graph paper, and relative areas were determined by counting squares. The yield of CIIIsb (Fabμ) amounted to approximately 55% of the original protein. On a molar basis, 2.3 moles of CIIIsb was produced per mole of reduced IgMsb.

Papain Digestion of IgM. IgM was hydrolyzed with 1% by weight of papain for 18 hr as described under the Methods section and the reaction mixture was applied to a Sephadex G-100 column and eluted with 0.15 M NaCl. The elution profile revealed two peaks; using antiserum 18, the first peak showed immunological identity with CIII but was deficient to CII and therefore designated as PIII (Figure 6A). The second peak was mostly dialyzable peptides.

The yield of PIII (Fabμ) amounted to approximately 40% of the undigested IgM. On a molar basis, 9.2 moles of PIII was generated per mole of IgM. The method of estimating the yield of PIII was similar to the method used for calculating the yield of CIIIsb from IgMsb.

After short-term (1.5 hr) digestion of IgM and application of the resulting digest to a Sephadex G-200 column equilibrated with 0.15 M NaCl, four peaks were obtained (Figure 7). Following the terminology used for the chymotrypsin C digestion products of IgM, these peaks were denoted as PI, PII, PIII, and peptides. PI, PII, and PIII after rechromatography were homogeneous by ultracentrifugal analysis and had sedimentation coefficients of 18.4, 7.0, and 3.7 S, respectively. The areas under each peak amounted to 13% for PI, 9% for PII, 34% for PIII, and 44% for peptides. However, the relative amounts of the various components varied somewhat using different batches of papain.

The Ouchterlony analysis clearly shows that the whole IgM, PI, and PII are antigenically equivalent but that PIII is antigenically deficient in μ-chain determinants (Figure 6B), using antiserum 18. The data on the quantitative complement fixation support and elaborate the results obtained by precipitation techniques (Figure 8). Both IgM and PI exhibited similar broad curves of complement fixation with respect to antiserum

TABLE I: Physical and Chemical Properties of Human IgM Fragments Produced by Enzymatic Hydrolysis.

	s^0_{20}	Mol Wt	% Hexose (g/100 g of protein)
IgM	18.5	845,000	5.8
IgMsb[a]	?	170,000	5.8
IgMsb[b]	7.1	123,000	
CI	16.6	733,000 and 606,000	
CII	6.6	135,000	2.2
CIII	3.8	40,000	
PI	18.4		
PII	7.0		
PIII (Fab)	3.7	37,000	1.6

[a] Obtained by reduction with 0.01 M dithiothreitol at pH 8.0. The plot of s *vs.* concentration for the reduced IgMsb was too scattered to permit the determination of its sedimentation coefficient. [b] Obtained by reduction with 0.01 M dithiothreitol at pH 8.0 and subsequently alkylated with a 10% excess of iodoacetamide.

18. Therefore, PI is most likely the unreduced and undigested IgM. The height of the PII curve indicates that this fraction has all the antigenic reactivity of undigested IgM and while it is very likely formed by reduction by the cysteine in the digestion mixture, it may be altered somewhat structurally compared with IgMsb produced by reduction in the absence of papain. This is indicated by the fact that PIII is more efficient and reaches the maximum in $C'H_{50}$ fixed at a lower antigen concentration than IgMsb. PIII upon immunoelectrophoresis using antiserum 1c (Figure 9) revealed a fast Fc-like fragment in addition to the slow-moving component similar to that in the 18-hr digest (as detected by rabbit 24 anti-L-chain serum). It appeared therefore that after shorter incubation times the third peak (PIII) contains two components of approximately equal size but differing significantly in electrophoretic mobility.

Unfortunately the reproducibility of short-term papain digestion to produce Fcμ fragment by this method was poor even with incubation time as short as 45 min and hence, the isolation of the fast fragment was not easily accomplished.

It was of importance to test experimentally our hypothesis that PII fragment resulted from the reduction of the polymer by 0.01 M cysteine under the condition of brief papain digestion. The IgM polymer was reduced by 0.01 M cysteine in pH 7.4 sodium phosphate buffer (ionic strength 0.1) for 90 min and subsequently alkylated by the equimolar concentration of iodoacetamide. The IgM and cysteine reduced mixture was scanned in a double-sector cell in an analytical ultracentrifuge. From peak areas estimates about 24% of the IgM polymer was reduced by 0.01 M cysteine to the 7S subunit.[2]

[2] To avoid the error due to the Johnston–Ogston effect, the values for the relative composition of the IgM polymer and monomer were extrapolated to infinite dilution by plotting them against total protein concentrations (4.5, 9.5, and 13.3 mg per ml).

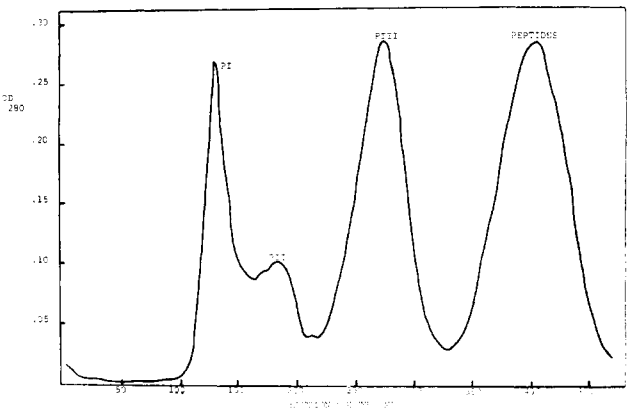

FIGURE 7: Elution pattern of the 1.5 hr papain digest of IgM from a Sephadex G-200 column (2.3 × 100 cm) equilibrated with 0.15 M NaCl.

Further Comparison of the Proteolytic Fragments of IgM. CII (6.6 S) was mildly reduced with 0.001 M dithiothreitol and subsequently alkylated by dialysis against 0.02 M iodoacetamide. Using rabbit 18 anti-IgM serum, the reductive product of CII (CIIr) was compared with CII and CIII by means of quantitative complement fixation. CII and CIIr are immunologically identical but CIII is deficient to CII and CIIr. CIIr appears as a single homogeneous peak of 4.4 S after isolation using Sephadex G-50 gel filtration. No peptides were released by reduction. It appears therefore that mild reduction does not destroy the antigenic determinant on the μ chain within CII (at least as seen with antiserum 18), although it significantly decreases molecular size.

Some physical and chemical properties of the IgM fragments produced by enzymatic hydrolysis are summarized in Table I.

Specificity of Antisera. Rabbit 18 antiserum exhibited the well-known individual (idiotypic) specificity directed toward the Fabμ fragment. Of the 9 μ-chain-specific antisera tested, only antiserum 18 made against the preparation of DiS IgM revealed a slow arc of Fab-like mobility upon immunoelectrophoresis of the chymotrypsin C digest of DiS IgMsb. Moreover, the Fabμ fragment derived from other Waldenstrom IgM preparations did not react with antiserum 18 as detected by gel precipitation. On screening of about 20 normal and Waldenstrom IgM preparations, it was found that nearly half

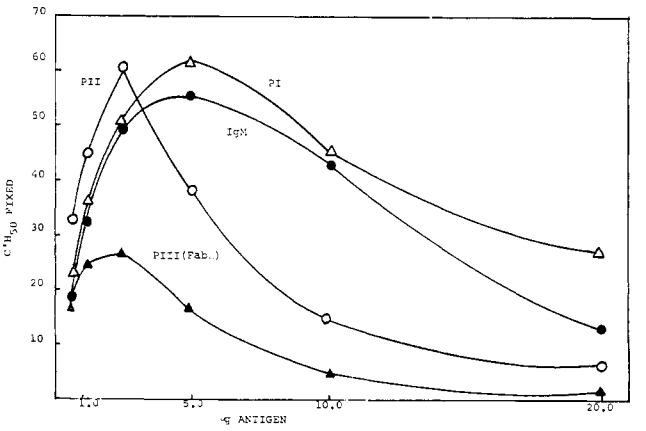

FIGURE 8: Fixation of complement by IgM and the papain fragments of IgM in the presence of antiserum 18 (anti-IgM) diluted 1/400.

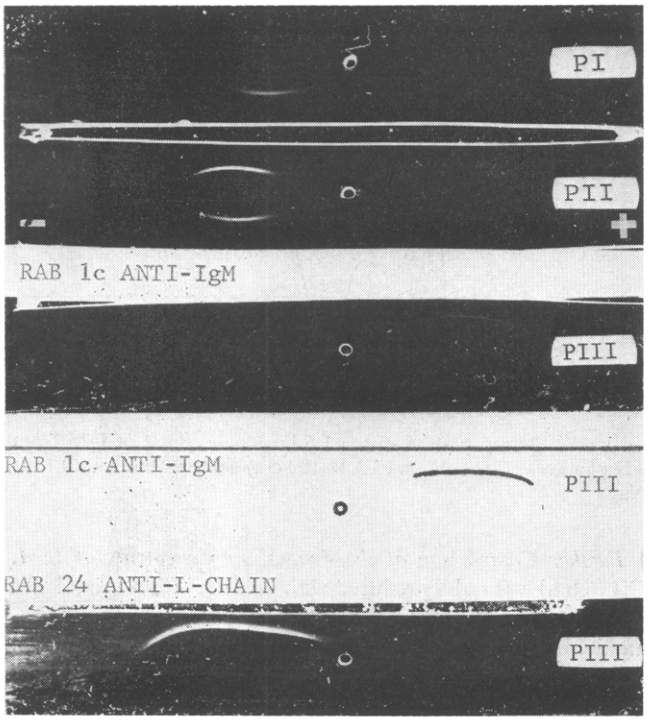

FIGURE 9: Immunoelectrophoretic pattern of the 1.5-hr papain digestion fragments of IgM against antiserum 1c (anti-IgM) and antiserum 24 (anti-L-chain).

of the complement-fixing activity of antiserum 18 was directed toward the individual specificity of DiS IgM.

DiS IgM was reduced and alkylated and its μ chain and L chain (λ type) were isolated by passage through a Sephadex G-100 column equilibrated with 1 N propionic acid. CIII which corresponds to Fabμ fragment and DiS λ chain, when compared by quantitative complement fixation, revealed marked differences in their ability to fix $C'H_{50}$. Inhibition of CIII reactivity toward antiserum 18 at higher concentrations of DiS λ chains demonstrated that approximately 30% of the Fabμ reactivity of antiserum 18 was directed toward isolated L chain and that the majority (approximately 70%) was specific for the Fabμ conformation.

Table II summarizes the specificities of some of the antisera used in our studies.

TABLE II: Specificities of Antisera.

Antiserum	IgM and IgMsb	CII	CIII and PIII (slow)	PIII (fast)
Rab 18	+	+	+	−
Rab 1c	+	−	−	+
Rab 71	+	+	−	+
Rab 74	+	−	−	−
Rab 24	+	+	+	−

Discussion

Chymotrypsin C digestion yields proteolytic fragments similar to but with certain differences from those produced by tryptic hydrolysis of IgM and IgMsb as described by Miller and Metzger (1966). This enzyme is differentiated from trypsin by its isoelectric point (4.1–4.2) which is well below that of trypsin (10.1) and in its substrate specificity. Chymotrypsin C rapidly hydrolyzes poly-L-glutamic acid into peptides in marked contrast to trypsin which does not attack this substrate (Gjessing and Hartnett, 1962).

A diagram depicting the possible relationship of chymotrypsin C fragments (CI, CII, and CIII) to the papain fragments (PI, PII, and PIII) is shown in Figure 10. A circular pentameric arrangement of IgM molecule suggested in this diagram although rather arbitrary is in accord with recent electron microscopic observations (Svehag *et al.*, 1967; Chesebro *et al.*, 1968). Furthermore, such a molecular architecture of IgM could account satisfactorily for the presence of a cysteine residue next to the C terminus of the μ chain (Doolittle *et al.*, 1966; Abel and Grey, 1967). It has been suggested that this cysteine residue might be involved in cross-linking between IgM subunits.

In consideration of the immunological data and molecular weights, it is postulated that chymotrypsin C cleavage takes place on both sides of the inter-μ-chain disulfide linkage and that the first enzymic cleavage splits off 6.6S fragment from IgM. Work is currently in progress to determine the number of inter-μ-chain disulfide bonds involved in the cleavage.

Recently Morris and Inman (1968) have reduced IgM under the gentle condition of 0.015 M mercaptoethylamine and alkylated with iodoacetic acid. Amino acid analysis on the resulting IgMsb showed a value of about two carboxymethylcysteine residues per molecule. This IgMsb appears to have maintained its original interchain disulfide bond integrity because it failed to dissociate in 1 N propionic acid into μ and L chains until subsequently reduced with 0.2 M 2-mercaptoethanol. These findings suggest that there are two intersubunit disulfide bonds per IgMsb which links the IgM subunit together.

It is believed that CIII with the molecular weight of 40,000 corresponds to an Fabμ fragment. The data also suggest that CII with a molecular weight of 135,000 is a dimer of CIII plus an additional fragment of 55,000 which is degraded into peptides after prolonged digestion. CI is a mixture which can be resolved by equilibrium ultracentrifugal techniques into two major components with weight-average molecular weights of 773,000 and 606,000. It seems probable, therefore, that CI consists of IgM molecules from which one or more $F(ab')_2\mu$ fragments have been hydrolyzed. Residual $F(ab')_2\mu$ fragments are still attached to the $(Fc\mu)_5$ portion of the IgM molecule since CI and IgM are antigenically identical.

It has been shown that blocking agents such as iodoacetamide or *N*-ethylmaleimide do not inhibit the digestion of human IgG by chymotrypsin C (Calvanico and Tomasi, 1967). Therefore, the possibility that these subunits are produced by reduction of the labile interchain disulfide bridge due to contamination with small amounts of reducing agents or through a disulfide-bond interchange mechanism seems unlikely.

Suzuki and Deutsch (1967) calculated the activation energy of IgM reduction to form subunits to be 5600 cal/mole for 0.03 M 2-mercaptoethanol. This value indicates that it is a relatively fast reaction. It is reasonable to suggest therefore that in the presence of 0.01 M cysteine (as is required for the activa-

tion of papain) IgM is reduced to IgMsb and then the 7S subunit is degraded into PIII by papain. PI is most likely undigested IgM and PII is an IgMsb. In the absence of papain, 24% of the IgM polymer is reduced by 0.01 M cysteine to the 7S subunit. Possibly an equilibrium exists between 19S IgM and the 7S monomer and this equilibrium shifts as the monomer is degraded by papain into PIII. This explanation could account for the combined yield of 87% for PII, PIII, and peptides.

The quantitative complement fixation data indicate that PII has been altered somewhat structurally since it is more efficient than IgMsb in the fixation reactions. One might also speculate that some peptide bonds within PII molecules have been cleaved but that the subunits are still held together by noncovalent forces and/or intrachain disulfide bonds. Here, one has to bear in mind that the reduced-alkylated IgM subunit exhibits the similar broad plateau as native IgM while PII resembles CI in its C'F characteristics (see Figures 4 and 8). The broad equivalence zone given by IgMsb is unexpected in view of the usual explanation for this type of plateau as being due to differences in size between the antigen and antibody. Based both on antigenic analysis and immunoelectrophoresis PIII appears to contain two components corresponding to Fabμ and Fcμ fragments of about equal size. From its elution position on Sephadex and the sedimentation coefficient of PIII (3.7 S) produced by short-term papain hydrolysis, the molecular weight of Fcμ fragment is probably of the order of 40,000.

Recently Mihaesco and Seligmann (1966, 1968) and also Onoue *et al.* (1967) have reported that the Fc portion of IgM is susceptible to papain digestion contrary to the experience with human IgG. This also has been our observation. After an 18-hr incubation with papain, PIII (Fabμ fragment) with a molecular weight of 37,000 is the only immunologically reactive fragment detectable. However, using an anti-μ-chain antiserum, Onoue *et al.* (1967) detected an electrophoretically fast Fcμ fragment in the short-term digestion product of IgMsb by papain. Our data indicate that a Fcμ fragment liberated by papain from IgMsb can also be detected by some anti-IgM antisera but that its production is erratic and that yields are small. The fact that high molecular weight $(Fc\mu)_3$ (160,000 molecular weight) and $(FC\mu)_5$ (320,000 molecular weight) fragments have been isolated by the methods which limit or eliminate the reductive activity of cysteine on IgM suggests the importance of the polymeric conformation in conferring stability to the Fcμ fragment following papain hydrolysis (Mihaesco and Seligmann, 1968; Onoue *et al.*, 1968a,b).

Chymotrypsin C cleavage apparently gives rise to relatively larger F(ab')$_2\mu$ fragment (135,000 molecular weight) as compared with that of tryptic hydrolysis (114,000 molecular weight) (Miller and Metzger, 1966). The considerable variation in the sites of first enzymic cleavages of IgM by enzymes such as chymotrypsin C, papain, pepsin (Kishimoto *et al.*, 1968), and trypsin is noteworthy. Moreover, the large amounts of dialyzable peptides produced after each enzyme digestion seem to indicate extended areas of unfolded structures within the IgM molecule which are susceptible to these enzymes. Mihaesco and Seligmann (1968) have demonstrated that the antigenic determinants against which their anti-IgM sera are directed are localized on this "papain-sensitive" region. They showed that their antisera against IgM after absorption with both Fabμ and Fcμ fragments still reacted with IgM.

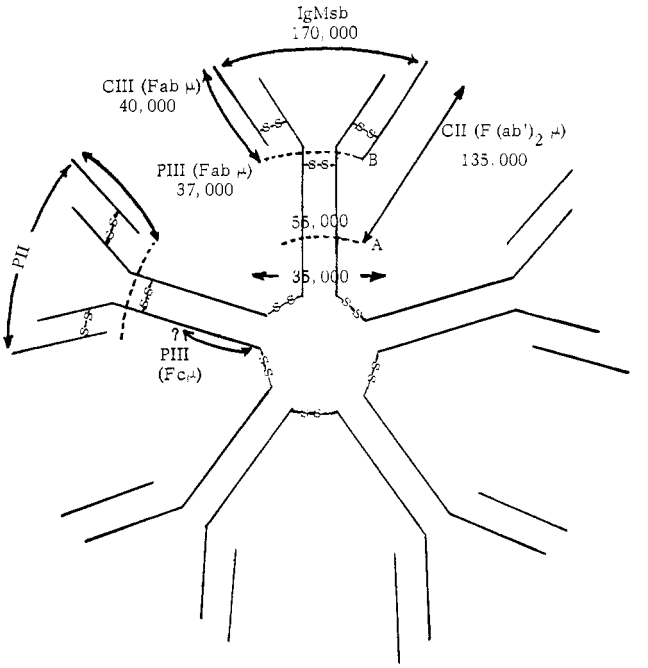

FIGURE 10: Diagram depicting the relationships of the chymotrypsin C and the papain fragments. A and B indicate the first and second cleavages of IgM by chymotrypsin C. PIII (Fcμ) represents a portion of μ chains in the C-terminal region beyond the primary papain cleavage site.

Numerous experiments have been carried out with chymotrypsin C hydrolysis of IgMsb by adjusting the time and conditions of enzyme digestion but have failed to produce an Fc-like fragment detectable by immunoelectrophoresis and/or disc electrophoresis. These results suggest that either all of the antisera we have used thus far lack antigenic markers on the C-terminal region of μ chain (beyond the first chymotrypsin C cleavage point), or more likely that the monomeric subunit of IgM does not yield a stable Fc-like fragment due to the extensive degradation of this region by the enzyme.

The carbohydrate (at least the hexose moieties) seems to be distributed over several areas of the μ chain. On a molar basis, CIII (Fabμ) contains 4% of the total hexose found in IgMsb and CII (F(ab')$_2\mu$) has 28% of the total hexose. Hence approximately one-third of the hexose is bound to CII and approximately two-thirds presumably resides on the C-terminal end of the first chymotrypsin C cleavage. It is interesting to note that $(Fc\mu)_5$ fragment (320,000 molecular weight) reported by Onoue *et al.* (1968a,b) contained about two-thirds of the total carbohydrate.

Antiserum 18 showed individual specificity directed toward the Fd piece and/or the apposition of the Fd piece and L chains and to a lesser extent toward the λ chain isolated from DiS IgM. As shown by complement fixation inhibition experiments, about 30% of the Fabμ reactivity of antiserum 18 is directed toward isolated L chain and the majority (approximately 70%) is specific for the Fabμ conformation of DiS IgM.

Previously Miller and Metzger (1966) proposed that the intact IgM molecule is composed of five IgG-like subunits linked by disulfide bonds and therefore IgM has potentially ten antibody combining sites. However, Suzuki and Deutsch (1967) reported that the reduced subunit of IgM contains two H and

three L chains. These conflicting reports may in part be due to the difficulty of obtaining accurate molecular weights on the reductive subunits of IgM. In the present study, the molecular weight of IgMsb determined for the reduced IgM subunit (reduced by 0.01 M dithiothreitol but not alkylated) was 170,000 which is about one-fifth of the molecular weight of IgM (845,000). However, the molecular weight determination on the reduced-alkylated IgMsb presented considerable problems because of heterogeneity. In general our data seem to parallel the observation by Suzuki and Deutsch (1967) that the reduced IgMsb loses L chains subsequent to alkylation. The reduced-alkylated IgMsb (by 0.01 M dithiothreitol) had an average molecule weight of 123,000 and since the reduced-unalkylated IgMsb had a molecular weight of 170,000, these data are consistent with the loss of two L chains as suggested by Suzuki and Deutsch. Further work is presently in progress to confirm and extend these initial findings.

Quantitative studies were carried out to ascertain the yields of Fabμ fragments from IgMsb by chymotrypsin C digestion and from IgM by papain hydrolysis. On a molar basis, 1 mole of IgMsb produced approximately 2 moles of Fabμ fragments while 1 molecule of IgM yielded nearly 10 Fabμ (PIII) units. These results suggest that each 19S molecule contains 10 Fabμ units and therefore presumably the potential for 10 antibody combining sites. However, it should be stressed that since no direct measurements were made of valency in this study, no statement can be made concerning the functional (antigen binding) capacity of the Fab units in the intact molecule.

These studies within experimental errors corroborate the findings by Miller and Metzger (1966) that IgM possesses potentially ten antibody combining sites. It is interesting to point out two recent reports. One by Frank and Humphrey (1968) indicating that rabbit anti-Forssman IgM antibody may have five effective antigen binding sites and five ineffective sites, and the other by Onoue *et al.* (1968a,b) suggesting that rabbit antihapten IgM antibody possesses five strong combining sites and five weak sites. Whether the ineffectiveness as antigen binding sites of the five "weak" H–L chain pairs within the IgM molecule is due to distinctive features in the primary structure of these chains or to steric factors inherent in the conformation of the intact IgM molecule requires further study.

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