The Cleavage of Human γ-Globulin by Papain*

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The hypothesis that antibody γ-globulin molecules consist of functionally different subunits had its origin in 1936 in the demonstration by Parvencev (1) that horse diphtheria antitoxin, when digested with pepsin, suffered no loss in antitoxic potency, whereas its antigenic activity was reduced. In the period from 1941 to 1946, much work was done on the partial enzymatic hydrolysis of antitoxic pseudoglobulins to give biologically active fragments (2, 3). Northrop (4, 5) published evidence for the crystallization of an active fragment of diphtheria antitoxin having an $s_{20,w}$ of 5.2 S and a molecular weight of 100,000. Petermann (6) reported that papain cleaved bovine serum pseudoglobulin into halves and quarters with an $s_{20,w}$ of 5.3 S and 3.7 S, respectively. Human γ-globulin (7) was similarly cleaved to yield subunits with a molecular weight of 47,000 and an $s_{20,w}$ of 4.1 S. However, a later analysis of the spreading boundary in the analytical ultracentrifuge by Williams et al. (8) indicated that the resolution into 3 S, 5 S, and 7 S components was rather arbitrary. Following earlier observations (9), Porter in 1958 (10) isolated three immunologically active fragments from a papain digest of rabbit γ-globulin antibody. These fragments (designated Fractions I, II, and III in the order of chromatographic elution) all had sedimentation coefficients of about 3.5 S, and molecular weights of 50,000, 53,000, and 80,000, respectively (11). Fractions I and II were similar in chemical and biological properties; each had the power to combine with, but not to precipitate, the antigen, and presumably each contained one antibody-combining site or valence. Fraction III had most of the antigenic specificity of the original molecule, but no antibody activity.

Because of the great importance of these observations for the study of the structure of antibodies, we have extended this approach to normal and pathological human γ-globulins and to the γ-globulins of various species. The kinetics and the mechanism of the specific cleavage have been studied. Two 3.5 S components, designated Fractions A and B, have been isolated from human γ-globulin and have been analyzed, and an hypothesis is presented to explain their sequential appearance.

EXPERIMENTAL PROCEDURE

Materials and Methods

Protein Specimens—Three types of purified protein specimens were studied in this investigation; namely, (a) normal human γ-globulin obtained from pooled plasma, (b) pathological γ-globulins obtained from the sera of patients who had multiple myeloma, and (c) commercial samples of the γ-globulins from various species. From the Merck Institute for Therapeutic Research, 7701 Burholme Avenue, Fox Chase, Philadelphia 11, Pennsylvania.

* The single sample of normal human γ-globulin used throughout this investigation was received from the Merck Institute for Therapeutic Research through the courtesy of Dr. Benjamin E. Sanders. This sample (Lot No. 45875) was prepared from pooled plasma as Fraction II by the ethanol fractionation method. Note that the earlier nomenclature for ethanol fractions of whole γ-globulin is not to be confused with Porter's designation for the fragments obtained by papain cleavage (10).

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‡ The myeloma γ-globulins used in this investigation have been described (12). These specimens (designated Wi, Jo, La, and Ag after the name of each patient) had been prepared by repeated ammonium sulfate precipitation from the serum of the individual patients. Considerable information had been accumulated on the properties of these proteins, such as sedimentation constants, electrophoretic mobilities, and $N$-terminal amino acids (12).

§ Sample of γ-globulins from various species were obtained from Pentex, Inc., Kankakee, Illinois, as ethanolic Fraction II. Most of these specimens were not free from contaminating proteins, such as γ1- or γ2-globulins, and several of the specimens had to be fractionated further by the use of chromatography (13).

1 The abbreviations used are: EDTA, ethylenediaminetetraacetate; CM, carboxymethyl; CMB, para-chloromercuribenzoate; DAP, 2,3-dimercaptopropanol (British anti lewisite); and DNP, dinitrophenyl.
was prewashed with mixtures of 0.9 M NaOH-0.5 M NaCl (1:1), followed by water, and was then equilibrated with the starting buffer. Since Type 20 is a fast flow grade, no decantation during washing, and hence, no air pressure during chromatography was necessary. To prevent overloading, the quantity of the ion exchanger used was at least 10 times that of the total protein to be chromatographed.

The sample to be chromatographed was dialyzed against the starting buffer before application to the column, which had also been equilibrated with the same buffer. The protein was collected either by gradient or stepwise elution. In the former case, a solution of higher ionic strength was introduced into a constant volume mixing flask (500 ml) containing the starting buffer. The stepwise elution method was employed when it was desired to collect the protein as a concentrated fraction in a small volume. This was achieved by eluting the column directly with a solution of higher ionic strength (i.e. 0.5 M NaCl). All chromatographic operations were carried out at room temperature as recommended by Porter (10, 11) to avoid precipitation of fractions in the cold. Crystallization was observed for the rabbit γ-globulin Fraction III, but not for the human protein cleavage products.

The protein concentration in the eluate was estimated by measuring the absorption in the Beckman DU spectrophotometer at 280 μm. When CMB was used as the inhibitor, the absorption of the eluate at 250 μm was also recorded; for at this wave length CMB has a high absorption, whereas the protein has a minimal absorption. The change of ionic strength upon gradient elution was followed by means of a conductivity bridge (Type 32163, LKB Produktor, Stockholm, Sweden). All conductivities and have a sharper chromatographic pattern than normal human γ-globulin (13, 17).

Ultracentrifugation—The Spinco model E analytical ultracentrifuge was employed for the estimation of the molecular homogeneity of the protein specimens and also for following the kinetics of cleavage of γ-globulins resulting from the action of papain. For sedimentation measurements, the solution was run at 59,780 r.p.m. in a standard cell with rotor temperature maintained at 20°. Because the action of papain was not inhibited in some experiments, the timing of the run and of the automatic photographs were made identical in all runs, and the relative area of the components is given in all cases for a period of 50 minutes after full speed was reached. The s20,w values reported were not corrected for the effect of protein and buffer concentration, because in most instances it was of interest to ascertain only whether cleavage occurred or not. The Yphantis-Waugh separation cell was employed for the separation of protein molecules which possess different sedimentation constants. With the use of this procedure to remove the undigested 6.6 S γ-globulin, Fraction A was purified for amino acid analysis.

Starch Gel Electrophoresis—The apparatus for starch gel electrophoresis was made of Plexiglas similar to the basic design given by Smithies (18, 19). The hydrolyzed starch was obtained from Connaught Medical Research Laboratories. The preparation, dyeing, and washing of the gel were performed as described by Smithies (18). In order to make the γ-globulins migrate sufficiently away from the origin, a 0.025 M sodium acetate buffer pH 5.5 was used to prepare the gel, and 0.05 M sodium acetate buffer, pH 5.5, was used for the buffer vessels of the electrode compartments. Electrophoresis was carried out at room temperature for 12 hours at 120 volts and 20 ma (5.6 volts per cm).

Amino Acid Analysis—The amino acid composition of the proteins and cleavage products was estimated by use of the Spinco automatic amino acid analyzer, model MS (20, 21). The instrument was initially calibrated with a known synthetic mixture of amino acids in order to obtain a constant for each amino acid. Frequent calibration was found to be necessary. The sample for amino acid analysis was taken up in triply distilled 6 N HCl, sealed under vacuum, and hydrolyzed for 22 hours at 105–107°. Determinations were made at least in duplicate whenever the sample size permitted. For comparative purposes, the results are expressed as the percentage of the total number of μmoles of amino acids recovered, exclusive of ammonia and tryptophan.

RESULTS

Chromatographic Separation of Cleavage Products

Rabbit γ-Globulin—Before the attempt to separate the papain cleavage products of normal human γ-globulin by chromatography, an attempt was made to repeat Porter’s work on the separation of a 16-hour papain digest of rabbit γ-globulin into three fractions of approximately equal amounts (10, 11). As a control, an aliquot was taken immediately after the addition of papain, dialyzed in the cold, and chromatographed under conditions (CM-cellulose, 0.01 M acetate buffer pH 5.5) identical to those used for the sample which was incubated for 16 hours. Since Porter had used dialysis to stop digestion after 16 hours of incubation, it was surprising to find that this control yielded four chromatographic components, a result indicating that papain was quite active, even at 4–5°, during the dialysis period. Chromatography of the sample incubated for 16 hours showed only three components. Another control was made by chromatography of the original protein without the addition of papain. By superimposing the conductivity gradient curves of these three chromatograms, it was demonstrated that three of the four peaks corresponded to Porter’s Fractions I, II, and III, the fourth peak corresponded to the original unhydrolyzed γ-globulin which disappeared after 16 hours of incubation. It was evident that the initial reactions involved in the papain cleavage of γ-globulins were much more rapid than anticipated.

Human γ-Globulin—When normal human γ-globulin was treated with papain, incubated for 16 to 30 hours at 37°, dialyzed, and chromatographed under conditions identical to those...
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FIG. 1. Chromatography of human γ-globulin and its papain-digestion products. The numbers at the peak of the curves represent the conductivity (10⁻⁴ ohm⁻¹) of the eluate at that point. ——, original human γ-globulin, not treated with papain; ..., human γ-globulin incubated with crystalline papain for 16 hours at 37° and dialyzed overnight. The chromatographic peaks are designated A and B.

for rabbit γ-globulin, no resolution into fractions was obtained. However, a broad peak was eluted at a lower conductivity than for undigested γ-globulin. This could indicate that the protein digest as a whole possessed a greater negative charge at pH 5.5 than the original protein, or that it consisted of smaller molecules. Various chromatographic conditions were investigated to achieve a resolution of a 16-hour papain digest of human γ-globulin comparable to that reported for rabbit γ-globulin. The factors which were varied included the pH gradient at constant ionic strength and the ionic strength gradient at various starting pH values. The best resolution thus far obtained was provided by the system illustrated in Fig. 1. For this, the protein was applied to the CM-cellulose column in a 0.01 M phosphate buffer at pH 6 and was eluted by increasing the ionic strength with NaCl according to the gradient indicated by the conductivity curve of Fig. 1. The pH remained constant. Although by use of this chromatographic system, only one major peak (A) was obtained from a 10-minute papain digest of human γ-globulin as compared to that reported for rabbit γ-globulin. The factors which were varied included the pH gradient at constant ionic strength and the ionic strength gradient at various starting pH values. The best resolution thus far obtained was provided by the system illustrated in Fig. 1. For this, the protein was applied to the CM-cellulose column in a 0.01 M phosphate buffer at pH 6 and was eluted by increasing the ionic strength with NaCl according to the gradient indicated by the conductivity curve of Fig. 1. The pH remained constant. Although by use of this chromatographic system, only one major peak (A) was obtained from a 10-minute papain digest of human γ-globulin, two peaks (A and B) were always resolved after prolonged incubation. Yet in both a 10-minute and a 16-hour digest, most of the γ-globulin sedimented as a 3.5 S component. In the 16-hour digest, the first peak eluted (B) amounted to about 30% of the nondialyzable material, and the second peak (A) comprised about 70%. The dialyzable peptides were eluted with the first peak; this comes off before the gradient is initiated and just after the hold-up volume of the column and thus represents unadsorbed material. Fraction B (as opposed to Peak B) is defined hereafter as the 3.5 S component remaining after dialysis of this first peak. The second peak (A) contained a 3.5 S component (designated Fraction A) together with any residual 6.6 S γ-globulin. No dialyzable material was eluted with this peak. Although Fraction A was incompletely separable by chromatography from the original 6.6 S globulin under any conditions tried (see Fig. 2, upper left), it differed from the undigested protein in having an s₂₀ = 3.5 S, in its electrophoretic properties, and in being eluted at a lower conductivity. Under standard chromatographic conditions similar to those of Fig. 1, the conductivity of the eluate for the maximum of a peak containing mainly Fraction A was 34 (±2) × 10⁻⁴ ohm⁻¹, whereas that of the original γ-globulin was about 54 × 10⁻⁴ ohm⁻¹. Fraction A has thus far been separated from residual 6.6 S γ-globulin only by use of the separation cell in the analytical ultracentrifuge (see Fig. 2, lower left). Kinetic studies on the rate and sequence of appearance of Fractions A and B are described subsequently.

When an undialyzed aliquot of the same digest illustrated in Fig. 1 was chromatographed, a large increase of the first peak (B) was obtained. However, about 60% of the 280 μ absorbing material of this first peak could be removed by dialysis, indicating that small fragments, probably mixtures of peptides, were present. A high base-line at the meniscus of the ultracentrifuge diagram of the undialyzed first peak eluted by chromatography indicated the presence of material sedimenting slowly at full speed (59,780 r.p.m.), in addition to the 3.5 S component remaining in a dialyzed aliquot (see Fig. 2, upper and lower right hand diagrams). This material could be separated from the 3.5 S Fraction B by stepwise elution chromatography beginning at pH 4.5. It was completely dialyzable. Analysis indicated that it contained peptides, but no free amino acids (see below).

When the papain digestion was carried out in the presence of 8 M urea, almost all the 280 μ absorbing material was dialyzable. Chromatography of the dialyzed reaction mixture gave only one small peak upon elution with the starting buffer, and nothing came off at the position at which the original protein should appear. Thus, when native protein is treated with papain, the
3.5 S fragments are relatively stable to further action of the enzyme, whereas the denatured protein tends to be cleaved into a whole series of random peptides.

Ultracentrifugal Analysis of Cleavage Products

Comparison of γ-Globulins of Different Species—In this series of analyses, the γ-globulins of various species were incubated for different lengths of time at 37° with crystalline papain in the presence of cysteine and EDTA, and the reaction mixtures were analyzed ultracentrifugally without the addition of an inhibitor. As always, the area analysis is given for 80 minutes after full speed was reached. The reported time of incubation (given in quotation marks in Table I) represents the actual incubation period at 37° before loading the ultracentrifugal cell and starting the run. However, since no inhibitor was used and the temperature of the rotor during the run was kept at 20°, the papain was still somewhat active during the period of about 2 hours which elapsed until the two components were separated from each other. Thus, in the case of normal pooled human γ-globulin, about three-fourths of the protein was cleaved to 3.5 S components, even though the actual 37° incubation period was marked as “0” hour (Table I). Successive analysis of aliquots of the incubation mixture showed that after the initial rapid cleavage, more than 30 hours of incubation at 37° were required to cleave the remaining protein into 3.5 S fragments and peptides. This experiment indicated the existence of a fraction comprising about 10% of the original protein, which was relatively resistant to papain.

The effects of crystalline papain on the γ-globulins of pooled normal human plasma and those from various species are compared in Table I. In the case of the animal globulins, only the “0”- and “4”-hour incubation mixtures were analyzed in the ultracentrifuge. The results indicated that γ-globulins from various species are cleaved at different rates. Like normal human γ-globulin, two-thirds of the rabbit γ-globulin molecules are cleaved to 3.5 S fragments within the period of ultracentrifugation, whereas more than 4 hours of incubation at 37° before the run were required for a similar change in the γ-globulins of the horse and cow. The porcine protein seemed to be very resistant, since no 3.5 S component was detected at 0 hours. However, after incubation at 37° for 4 hours, an intermediate 5.5 S component and some 3.5 S material were formed.

This difference in susceptibility to papain cleavage of the γ-globulins of various species suggested that further study of the 3.5 S fragments of human γ-globulin was warranted before the concept of a tripartite structure suggested for rabbit γ-globulin (11) could be extended to the human protein.

Cleavage of Normal and Pathological Human γ-Globulins—A similar comparison was made of the effect of crystalline papain on normal and pathological human γ-globulins (Table I). The

<table>
<thead>
<tr>
<th>Species</th>
<th>γ-Globulin</th>
<th>&quot;0&quot; hr</th>
<th>&quot;4&quot; hr</th>
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<tr>
<td>Human</td>
<td>74</td>
<td>68</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit</td>
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<tr>
<td>Porcine</td>
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<td>42</td>
<td>22</td>
</tr>
<tr>
<td>Myeloma†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WI</td>
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<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Ag</td>
<td>90</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

* The reaction mixture consisted of 10 mg of γ-globulin in 1 ml of phosphate buffer (0.1 M, pH 6.0) containing 0.01 M cysteine and 0.002 M EDTA. The proportion of 1 mg of enzyme to 100 mg of protein was maintained in all cases. No inhibitor was employed.
† "0" and "4" hours designate the incubation period at 37° before loading the ultracentrifugal cell and starting the analysis.
‡ The myeloma globulins all had an s20, w 6.6 S, but specimen Jo had a mobility intermediate between γ- and β-globulins, whereas proteins WI and AG had a mobility at pH 8.6 identical to that of normal γ-globulin (12).
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MOLAR CONCENTRATION OF K$_3$Fe(CN)$_6$

FIG. 3. Ferricyanide inhibition of papain cleavage of human $\gamma$-globulin. The reaction mixture consisted of 10 mg of $\gamma$-globulin and 0.1 mg of papain in 1 ml of phosphate buffer (0.1 M, pH 6) containing 0.01 M cysteine and 0.002 M EDTA. The K$_3$Fe(CN)$_6$ was added to the reaction mixture at the final concentration indicated on the abscissa. Ultracentrifugal analyses were carried out immediately after the addition of all the reagents. In one series of experiments (represented by Curve II), the inhibitor was added to the mixture before the papain; in the other series (I), the enzyme was added first.

Effect of Activators and Inhibitors of Papain

Potassium Ferricyanide—It has been known for a long time that papain is activated by reducing agents, such as H$_2$S, HCN, cysteine, etc., and that its action is inhibited by sulfhydryl-combining reagents, such as CMB and iodoacetate (22). Since the effect of papain on $\gamma$-globulins was so very rapid and seemed specific, it was of interest to try to stop its action during the early stages to permit study of the products formed. Various inhibitors were investigated, beginning with potassium ferricyanide, which was recommended by Nisonoff and Woernley (23). Experiments were made with different concentrations of this chemical added to the reaction mixture before or after the addition of papain, and the reaction mixtures were studied immediately thereafter in the analytical ultracentrifuge. When the inhibitor was added to the reaction mixture at a concentration of 0.01 M immediately after the addition of papain, no inhibition was observed, but less 3.5 $S$ component was formed as the concentration of K$_3$Fe(CN)$_6$ was increased (Fig. 3, Curve I). When the inhibitor was added to the reaction mixture before the addition of papain, greater inhibition was observed (Fig. 3, Curve II), but the reaction was not completely stopped, even at a final concentration of 0.1 M K$_3$Fe(CN)$_6$. Since the order of addition of the reagents affected the degree of inhibition, it was clear that the cleavage reaction occurred very rapidly. Furthermore, K$_3$Fe(CN)$_6$ was not a suitable inhibitor for kinetic studies.

para-Chloromercuribenzoate—It has been suggested by Kimmel and Smith (22) that cysteine and EDTA can be replaced by BAL which acts as both a chelating and a reducing agent. Accordingly, this chemical was tried as the activator, and CMB was used to stop the reaction by blocking the sulfhydryl groups of both papain and BAL. Preliminary tests revealed that BAL did not activate papain as readily as the combination of cysteine and EDTA, although CMB stopped the proteolytic reaction completely. Furthermore, the addition of CMB to the BAL-activated digest resulted in heavy precipitation, together with the formation of aggregates, as shown by ultracentrifugal analysis. For these reasons, and because there was some doubt about the quality of the reagent, the use of BAL was abandoned.

It is well known that CMB, which is an excellent reagent for sulfhydryl groups, inhibits papain completely (22). However, because of the presence of cysteine at a concentration of 0.01 M in the standard reaction mixture, addition of CMB resulted in heavy precipitation. It was reasoned that if most of the cysteine were removed, the quantity of CMB necessary to inhibit the action of papain would be reduced. Attempts were thus made to wash off the cysteine through a CM-cellulose column before the addition of CMB, although CMB stopped the papain directly on the column.

Representative data are given in Table II for the chromatographic separation of the products of the cleavage of human $\gamma$-globulin by crystalline papain with CMB added to the column to inhibit the enzyme. Also given are some ultracentrifugal data...
on separate samples which were analyzed without the addition of an inhibitor. It should be recognized that there was a time lag of about 15 to 30 minutes for the application of the sample to the column in the case of chromatography and a delay of about 2 hours from the time of filling the cell until the two components were completely separated in the case of ultracentrifugation. It was obvious that more rapid methods to stop the cleavage reaction were needed. However, the data revealed a progressive increase in the proportion of the peptides which does not parallel the increase in the 3.5 S component. The results also appeared to indicate that Fraction B is formed after Fraction A, and possibly at the expense of the latter. Accordingly, a more rapid method of inactivating the papain was sought. In order to minimize nonspecific proteolysis, activated mercuripapain replaced crystalline papain in all subsequent kinetic experiments.

In further exploratory experiments using the ultracentrifugal pattern as a criterion, it was found that when the concentration of cysteine was reduced to 0.001 M, and when mercuripapain was substituted for crystalline papain, the reaction could be stopped within 10 seconds by the addition of CMB at a final concentration of 0.001 M. No heavy precipitation resulted in this case, and the final solution was suitable for ultracentrifugal and chromatographic analysis. With the use of this procedure, a series of experiments was performed as described below. Hereafter, a concentration of 0.001 M cysteine will be understood whenever CMB is to be used as an inhibitor.

**Kinetic Study of Cleavage of Human γ-Globulin by Mercuripapain Followed by CMB Inhibition**

**Chromatographic and Ultracentrifugal Analyses**—To study the efficacy of CMB as an inhibitor, an experiment was set up in which aliquots of the digest were taken and added to CMB solution after incubation for periods of from 0 to 10 minutes. Ultracentrifugal analysis of these aliquots revealed that 4% of the protein molecules were cleaved into the 3.5 S components, even when the inhibitor was added within seconds after the addition of papain. After 10 minutes of incubation, 80% of the protein was cleaved into the 3.5 S components, a reaction which thereafter increased very slowly (Fig. 4). On the other hand, in separate experiments, it was established that continued incubation after the addition of CMB produced no further change in the proportion of 3.5 S components. The results shown in Fig. 4 emphasize that the cleavage of human γ-globulin is nearly complete within minutes under conditions used by other workers who studied the products obtained after 16 hours of incubation.

In another experiment, a large quantity of human γ-globulin (1.0 g) was incubated with mercuripapain in 0.01 M phosphate buffer pH 6.0, containing 0.001 M cysteine and 0.002 M EDTA. After 0, 1, 3, 5, 8, 9, 24 1/2, 34, and 48 hours of incubation at 37°, a large aliquot was withdrawn and immediately added to a tube containing CMB to yield a final concentration of 0.001 M inhibitor. Chromatography by the stepwise elution method, ultracentrifugation, starch gel electrophoresis, and amino acid analysis were carried out on some of these aliquots. The relative amount of 3.5 S fragments versus the 6.6 S original protein, as obtained through sedimentation studies, and also the amounts

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**TABLE II**

**Chromatographic and ultracentrifugal analysis of products of cleavage of human γ-globulin by crystalline papain**

<table>
<thead>
<tr>
<th>Incubation period</th>
<th>Ultracentrifugal analysis</th>
<th>Chromatographic analysis*</th>
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<tr>
<td></td>
<td>3.5 S</td>
<td>6.6 S</td>
</tr>
<tr>
<td>hrs</td>
<td>%</td>
<td>%</td>
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<tr>
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<td>91</td>
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<td>8</td>
<td>94</td>
<td>6</td>
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</table>

* All figures are expressed as the percentage of the 280 mυ absorption of the total undialyzed hydrolysate. Any deviation of the sum from 100% is attributable to incomplete chromatographic recovery or volume change on dialysis. The inhibitor, CMB, was added on the column.

† The undialyzed hydrolysate was run on the column. The material eluted in the first peak (containing both peptides and Fraction B) was dialyzed, and the amount of peptides was obtained by subtracting the nondialyzable Fraction B from the first fraction.

The chromatographically isolated Fractions A and B, from the aliquots after 9, 24 1/2, and 48 hours of incubation, were concentrated, dialyzed, and analyzed by starch gel electrophoresis. For comparison, samples of the original human γ-globulin and the whole papain digest were also run under identical conditions (Fig. 6). Both the whole γ-globulin and the digest migrated as a broad band toward the cathode at pH 5.5. The component in the digest that moved more rapidly than whole γ-globulin proved to be Fraction A. Fraction B migrated as a sharper band than Fraction A, but more slowly at this pH. Because of the manner in which the aliquots were taken, the relative density of the bands is only approximately proportional to the abundance of the fraction in the digest at the time indicated. However, it is significant that the distance of migration and the length of the band are constant with time for each fraction. In many experiments, Fractions A and B, isolated by the column method and then dialyzed, have consisted predominantly of 3.5 S components and have the electrophoretic characteristics indicated in Fig. 6. Ultracentrifugal analyses of Fraction A from chromatographic runs CM-62A and CM-63A have already been shown in Fig. 2.

The above results (Figs. 4 to 6) indicated that human γ-globulin is cleaved very rapidly by papain into at least two 3.5 S fragments with characteristic chromatographic and electrophoretic properties. Within 10 minutes of incubation, about 80% of the protein was cleaved, whereas the remaining 20% was cleaved at a much slower rate. About 10% of the protein was

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rather resistant to cleavage by papain. The 3.5 S fragments that were formed during a short incubation period (i.e. within several hours) behaved chromatographically and electrophoretically like Fraction A (Figs. 5 and 6). During this period, very little Fraction B, which has a different chromatographic and

electrophoretic pattern, was produced. (Most of the undialyzed Fraction B at 30 minutes in Fig. 5 represents peptides and other unadsorbed nonprotein material.) After the initial rapid cleavage, Fraction A appeared to diminish gradually, whereas Fraction B increased proportionately, as if part of Fraction A had been converted to B until a constant value was reached. In this case, it was noted that the final amount of Fraction A was about twice that of Fraction B without correction for peptide material.

Stability of Fractions A and B—Since Fraction B was not formed during a short incubation, which still cleared 80% of the γ-globulin to 3.5 S fragments, it seemed necessary to test whether Fraction A was actually converted to B (or vice versa) when the isolated fractions were subjected to papain action. A series of successive digestion experiments was also performed to ascertain whether the papain or activator present had been insufficient to achieve complete conversion in the earlier experiments. For this study, a large sample of human γ-globulin was incubated with mercuripapain in the presence of cysteine and EDTA. After 24 hours of incubation, part of the reaction mixture was chromatographed to isolate Fractions A and B, and the latter were separately incubated with mercuripapain for 24 hours (left-hand diagram of Fig. 7). These reaction mixtures were again chromatographed to isolate the remaining Fractions A and B. Ultracentrifugal analysis was performed to estimate the quantity of the residual 6.6 S γ-globulin. As shown in the left-hand side of Fig. 7, further incubation of either Fraction A or B resulted in no interconversion of the fractions. In both cases, considerably more peptide was formed after additional incubation with papain. To test further whether additional papain and activators were required for the supposed conversion of Fraction A to B, an aliquot of the original 24-hour digestion mixture was incubated again for 24 hours with more cysteine and papain added to it (right-hand diagram of Fig. 7). Chromatographic analysis of this double incubation mixture showed the same distribution of Fractions A and B as in the single incubation, although more peptides were formed in the double incubation. When the amount of Fraction A was corrected for the content of 6.6 S γ-globulin, the ratio of Fraction A to Fraction B in both experiments was very close to 2:1. Fraction A obtained from the 48-hour double incubation mixture was reincubated with activated mercuripapain. Although the peptide material again increased, no new Fraction B was formed. Thus, the stability of Fraction A obtained after long incubation was further confirmed; i.e. it was not converted to Fraction B, in contrast to what appeared to happen to some of the material from Peak A obtained after short incubation.

The above experiments proved that only part of the early formed 3.5 S material of Peak A is converted to Fraction B and that the remaining 3.5 S material is Fraction A, which is resistant to prolonged papain digestion even in the presence of additional cysteine and papain. Fraction B seemed somewhat more susceptible to proteolytic degradation than did Fraction A, but the quantity of peptides increased considerably after prolonged papain action, whether on the whole digest or the isolated fractions.

Since the amount of peptide was quite variable in different experiments and seemed possibly unrelated to the cleavage reaction, we were led to investigate whether the action of mercuripapain alone without any activator would reduce the amount of peptides formed. The results given in Table III indicate that when human γ-globulin was incubated for 15 hours with mer-
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**FIG. 6.** Starch gel electrophoresis of whole human γ-globulin, an unfractonated papain digest of human γ-globulin, and chromatographically separated Fractions A and B. The cathode is to the left in the diagram. The conditions and time of electrophoresis were the same for all samples. CM-60, etc., are numbers of chromatography runs; A and B represent the respective fractions. The fractions analyzed were from the experiment illustrated in Fig. 5. For ultracentrifugal diagrams of Fraction A in chromatographic runs CM-62A and CM-63A, see Fig. 2.

**FIG. 7.** Flow diagram for an experiment involving successive incubation with activated mercuripapain of human γ-globulin and of the 3.5 S fragments thereby obtained.

Mercuripapain without any activator, very little peptide was formed, although 63% of the protein was cleaved to 3.5 S fragments. Chromatographic analysis in a separate experiment in which unactivated mercuripapain was incubated for 16 hours with γ-globulin revealed the presence of both Fractions A and B, as well as some of the original protein. In the presence of both cysteine and EDTA, only 6% of the original protein remained, but a large quantity of peptides was liberated at the same time. When either cysteine or EDTA alone was used as the activator, 80% of the protein was cleaved to 3.5 S fragments, and relatively little peptide was formed. Thus, mercuripapain is somewhat active in the absence of an added activator; full activity is achieved only when both cysteine and EDTA are present, but then a much larger proportion of peptides is formed.

**TABLE III**

**Effect of activators on cleavage of human γ-globulin by mercuripapain**

Incubation of 1% γ-globulin for 15 hours with mercuripapain in 0.01 M phosphate buffer pH 6.2 in presence of activators. The complete system contained 0.001 M cysteine and 0.002 M EDTA.

<table>
<thead>
<tr>
<th>Activators</th>
<th>Ultracentrifugal components</th>
<th>Trichloroacetic acid-soluble supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.5 S</td>
<td>6.6 S</td>
</tr>
<tr>
<td>Cysteine + EDTA</td>
<td>94</td>
<td>6</td>
</tr>
<tr>
<td>Cysteine</td>
<td>81</td>
<td>19</td>
</tr>
<tr>
<td>EDTA</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>None</td>
<td>63</td>
<td>37</td>
</tr>
</tbody>
</table>

* Proportion of components resolved by ultracentrifugal analysis.
† Percentage of material soluble in 5% trichloroacetic acid as measured by optical density at 280 μm.
‡ Calculated from ninhydrin determination, expressed as the number of leucine equivalents per mole of original protein.
It was interesting that when unactivated mercuripapain cleaved two-thirds of the protein molecules, only about seven bonds were broken per mole of original $\gamma$-globulin, whereas use of the fully activated enzyme led to a great increase in the number of bonds broken relative to the additional $\gamma$-globulin cleaved. Of course, calculation by this method of the number of bonds broken gives a composite value for any specific proteolytic cleavage to form 3.5 S units plus any random degradation of these units (or of the resistant 6.6 S fraction) to yield low molecular weight peptides. Thus, doubling the amount of soluble peptides by use of EDTA as an activator doubled the apparent number of bonds broken. It is conceivable that only one to two peptides by use of EDTA as an activator doubled the apparent cleavage to form 3.5 S units plus any random degradation of these units (or of the resistant 6.6 S fraction) to yield low molecular weight peptides. It was interesting that when unactivated mercuripapain seemed to be very rapid and specific, attempts were made to ascertain the nature of the specific cleavage; i.e., whether free amino acids or specific peptides, as well as the 3.5 S components, were released by enzymatic action, or whether only the 3.5 S fragments are formed in the cleavage of the original 6.6 S molecule. Because papain is known to be an enzyme with broad specificity (22), it would be surprising if only two peptide bonds were cleaved, the minimum necessary to form three fragments from a single polypeptide chain (e.g., rabbit $\gamma$-globulin); or if only one peptide bond were split, a possibility for human $\gamma$-globulin, which has at least two polypeptide chains presumably joined together by disulfide bonds which might be exposed in the digest.

The presence of any free amino acids liberated during the initial cleavage was investigated for in the case of an activated mercuripapain digest which was inhibited with CMB after 10 minutes of incubation. The protein was precipitated with trichloroacetic acid. The supernatant solution was extracted with ether to remove trichloroacetic acid; it was then concentrated, and an aliquot was analyzed directly for amino acids without hydrolysis. The results indicated that no free amino acids were present in the acid-soluble fraction, whereas all 17 amino acids that survive acid hydrolysis of $\gamma$-globulin were found when an aliquot of the trichloroacetic acid-soluble fraction was hydrolyzed with HCl (Table IV). Since precipitation of protein by trichloroacetic acid may not be 100% complete, any trace amount of protein present in the supernatant solution may obscure the amino acid composition of the peptides, the more so since after short incubation the peptides represent only about 5% of the original protein. Hence, the chromatographic method was applied to collect the peptide material from a similar 10-minute digest by the use of a CM-cellulose column equilibrated at pH 4.5. At this pH, the peptides, together with other small molecules, such as CMB, cysteine, and EDTA, are eluted, whereas Fraction B is bonded to the column. The eluted peptide fraction was completely dialyzable, and like the trichloroacetic acid supernatant solution, it contained all the amino acids found in an acid hydrolysate of the whole $\gamma$-globulin (Table IV). Thus, the presence of a mixture of peptides was indicated because it is unlikely that all 17 amino acids are linked in one polypeptide chain that is small enough to be dialyzable.

Because the presence of added cysteine in the peptide material obscured the quantity of cysteine covalently linked in the peptides, the gel filtration method using Sephadex (25) was applied to separate the small molecules (CMB, cysteine, EDTA, and phosphate ions) from the true peptides. The results indicated that although the main peptide peak (P-1) was well separated from the CMB-cysteine peak, a tailing part (P-2) was also present. The relative increase in ninhydrin color after hydrolysis proved that the tailing peak consisted of peptides of shorter chain length than those in the main peptide peak. Amino acid analysis of both Sephadex fractions indicated the presence of the same 17 amino acids. Thus, further confirmation was obtained for the liberation of a mixture of peptides during 10 minutes of incubation with activated mercuripapain.

Although the qualitative composition is similar for the various peptide fractions and the whole protein, there are marked differences in the quantitative composition. The capacity of the Sephadex column (G-25) to separate a mixture of peptides was established by a test run on a tryptic digest of serum albumin with added EDTA, cysteine, and CMB as in the papain digest. The CMB-cysteine and peptide peaks were located by measurement of the absorption at 280 and 250 m$\mu$, the conductivity, and the ninhydrin color. The first fraction passing through (analogous to P-1 of Table IV) gave a five-fold increase in ninhydrin color after HCl hydrolysis compared to only a doubling in color for a fraction analogous to P-2. Thus, the latter contains the smaller peptides, as expected. An attempt was also made to use Sephadex with a higher percentage of water regain (G-75) for separation of 3.5 S and 6.6 S material. However, since in a trial run ovalbumin ($\varepsilon_{280} = 3.4$ S) and $\gamma$-globulin (6.6 S) were eluted together, the procedure did not seem feasible for the separation of $\gamma$-globulin and the 3.5 S fractions.

### Table IV

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Trichloroacetic acid supernatant</th>
<th>Peptide mixture*</th>
<th>Peptide fractions*</th>
<th>$\gamma$-Globulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Lysine</td>
<td>5.35</td>
<td>2.26</td>
<td>2.73</td>
<td>3.65</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.58</td>
<td>1.04</td>
<td>1.49</td>
<td>1.20</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.00</td>
<td>1.10</td>
<td>1.74</td>
<td>1.83</td>
</tr>
<tr>
<td>Aspartic</td>
<td>8.56</td>
<td>9.42</td>
<td>11.38</td>
<td>7.83</td>
</tr>
<tr>
<td>Threonine</td>
<td>9.62</td>
<td>8.87</td>
<td>7.79</td>
<td>10.32</td>
</tr>
<tr>
<td>Serine</td>
<td>12.21</td>
<td>12.32</td>
<td>8.43</td>
<td>13.05</td>
</tr>
<tr>
<td>Glutamine</td>
<td>10.30</td>
<td>11.09</td>
<td>16.43</td>
<td>10.42</td>
</tr>
<tr>
<td>Proline</td>
<td>11.90</td>
<td>9.28</td>
<td>10.38</td>
<td>9.23</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.27</td>
<td>7.90</td>
<td>4.74</td>
<td>8.63</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.76</td>
<td>0.60</td>
<td>7.40</td>
<td>0.62</td>
</tr>
<tr>
<td>Valine</td>
<td>4.27</td>
<td>8.24</td>
<td>7.07</td>
<td>10.21</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.91</td>
<td>0.67</td>
<td>0.28</td>
<td>0.65</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.32</td>
<td>2.40</td>
<td>1.79</td>
<td>2.42</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.35</td>
<td>8.75</td>
<td>5.12</td>
<td>9.57</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.90</td>
<td>4.29</td>
<td>2.46</td>
<td>1.41</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.51</td>
<td>4.14</td>
<td>4.96</td>
<td>2.63</td>
</tr>
</tbody>
</table>

* The peptide mixture was prepared by chromatography of the papain digest of $\gamma$-globulin, and the peptide fractions P-1 and P-2 were obtained from the mixture by the gel filtration method (see the text).
Amino acid composition of fractions A and B of normal γ-globulin

Expressed as the percentage of the total recovered moles of amino acids, exclusive of ammonia and tryptophan.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Fraction A</th>
<th>Fraction B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16 hrs</td>
<td>25 hrs</td>
</tr>
<tr>
<td></td>
<td>(1)</td>
<td>(2)</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.42</td>
<td>6.59</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.79</td>
<td>1.82</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.09</td>
<td>3.15</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>7.87</td>
<td>7.90</td>
</tr>
<tr>
<td>Threonine</td>
<td>8.38</td>
<td>8.39</td>
</tr>
<tr>
<td>Serine</td>
<td>13.01</td>
<td>13.10</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9.77</td>
<td>9.70</td>
</tr>
<tr>
<td>Proline</td>
<td>6.99</td>
<td>6.82</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.34</td>
<td>7.30</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.79</td>
<td>5.74</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>2.22</td>
<td>2.36</td>
</tr>
<tr>
<td>Valine</td>
<td>8.88</td>
<td>8.91</td>
</tr>
<tr>
<td>Methionine†</td>
<td>0.63</td>
<td>0.82</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.12</td>
<td>2.20</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.66</td>
<td>7.72</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.38</td>
<td>4.19</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.05%</td>
<td>3.48%</td>
</tr>
</tbody>
</table>

* Single analysis. All other results are in duplicate.
† Includes methionine sulfoxides.
‡ Includes glucosamine.

In these experiments the proportion of Fraction B in the dialyzed digest was only 9 to 12% and the ratio of Fraction A to B after correction for the 0.6 S component was greater than the value of 2.0 observed earlier. The proportion of peptides was increased, and it was thought that some reactivation of papain had occurred during the unusually long dialysis period, with a resultant preferential degradation of Fraction B. To establish that Fraction A eluted with 0.5 M NaCl by the stepwise method was free from Fraction B and was analogous to the 3.5 S fraction identified by the gradient method (see Fig. 1), a sample of Fraction A from a 48-hour digest was rechromatographed by the gradient method. None of the protein came off before the gradient was applied, and most was eluted at the same conductivity as the Fraction A shown in Fig. 1 (i.e. 34 X 10^-4 ohms^-1). This indicates that a true separation from Fraction B was obtained by the stepwise method. However, a minor component with the low optical density ratio of 0.45 (280 μA to 250 μA) came off the column just before the conductivity of the eluent began to increase. This material (not indicated in Fig. 1 in which CMB was not used) proved to be CMB-papain, for in a control run the latter was eluted at the same position and had the optical density ratio given above. Since papain was present in the original digest at an enzyme to protein ratio of only 1:100, the CMB contaminant represented no more than 2 to 3% of Fraction A.
The amino acid composition of these fractions was analyzed, and the average of duplicate values is given in the first three columns of figures in Table V. The close agreement of the composition of these fractions is shown by the standard deviation given in the fourth column. In all cases, the standard deviation is within the range of error (+3%) attributed to the method (20, 21), except for proline and methionine; in the latter case, the amino acid is present in small amount and is partially oxidized during acid hydrolysis. For comparison, the amino acid composition of the original human γ-globulin is shown in the fifth column. The resemblance of Fraction A to the whole γ-globulin is striking. Probably, only the decrease in dicarboxylic acids and proline and the increase in hydroxyamino acids are significant. A decrease in dicarboxylic acids would explain the more rapid cathodic mobility of Fraction A at pH 5.5, i.e., as the result of a greater net positive charge. Despite this, and perhaps as a result of its smaller size, Fraction A is eluted at a lower conductivity than intact γ-globulin from the negatively charged CM-cellulose column at pH 6.0. Since chromatographically isolated Fraction A contains about 10% of residual 6.6 S γ-globulin, an attempt was made to remove the latter by ultracentrifugation in the separation cell. In a separate experiment, a sample of Fraction A (48-hour incubation) was purified by this method; the amino acid composition is given in the sixth column of Table V. The analogy to whole γ-globulin is again evident. As in the series of analyses on chromatographically isolated Fraction A, the ultracentrifugally purified Fraction A was lower in dicarboxylic acids and higher in serine than the whole protein. However, lysine and proline were anomalously high. Unfortunately, the ultracentrifugal method was too tedious to prepare an adequate number of samples for repeated analysis.

Because Fraction B is more susceptible than Fraction A to proteolytic digestion, one might expect some variation in the composition of Fraction B after different periods of incubation. The results given in the last two columns of Table V suggest that Fraction B isolated after 24 and 48 hours of incubation has a different composition. Since these data represent single analyses on samples obtained in different experiments, they serve only in a general way to indicate an increase in dicarboxylic acids relative to whole γ-globulin or Fraction A. Such an increase would explain the lower cathodic mobility of Fraction B at pH 5.5 and the failure of Fraction B to be adsorbed to the negative CM-column at pH 6.0.

Table VI
N-Terminal groups of dialyzed papain digests of human γ-globulin

Results are expressed as the optical density ratio of the DNP-amino acid relative to DNP-aspartic acid. The latter was present in a molar ratio of about 1.0 in the original protein and in all the incubated samples. Traces of DNP-serine were present in all samples.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Aspartic acid</th>
<th>Glutamic acid</th>
<th>Threonine</th>
<th>Leucine or isoleucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min</td>
<td>1.00</td>
<td>1.45</td>
<td>1.17</td>
<td>0.31</td>
</tr>
<tr>
<td>7 hr</td>
<td>1.00</td>
<td>1.46</td>
<td>0.44</td>
<td>1.46</td>
</tr>
<tr>
<td>16 hr</td>
<td>1.00</td>
<td>1.24</td>
<td>*</td>
<td>1.29</td>
</tr>
<tr>
<td>25 hr</td>
<td>1.00</td>
<td>1.28</td>
<td>*</td>
<td>1.07</td>
</tr>
<tr>
<td>48 hr</td>
<td>1.00</td>
<td>1.54</td>
<td>*</td>
<td>1.42</td>
</tr>
</tbody>
</table>

* Negligible.

Amino End Group Determination—Aliquots of the digests from which Fraction A was isolated for amino acid analysis (Table V) were taken for N-terminal group estimation according to the method of Sanger (26). Before reaction with fluorodinitrobenzene, the digests were dialyzed to remove peptides, but were not fractionated by chromatography. The ether-soluble DNP-amino acids liberated on acid hydrolysis were separated by paper chromatography by the one-dimensional method of Blackburn and Lowther (27) with the use of tertiary amyl alcohol and pldululate buffer. In two-dimensional chromatography, the latter system was used in the first direction; for the second dimension, 1.5 M phosphate pH 6 was employed (28). Water-soluble DNP-amino acids were separated by use of the tertiary amyl alcohol system. The determination was semiquantitative because of transfer errors and the lability of DNP-amino acids; hence, the results were expressed as the molar ratio relative to DNP-aspartic acid, which was present in the original γ-globulin in a molar ratio of about 1.0. From the data of Table VI it is evident that the proportion of aspartic and glutamic acid end groups did not change significantly upon papain cleavage of γ-globulin. However, after 10 minutes of incubation, a new amino end group, threonine, appeared in stoichiometric proportions relative to the aspartyl end group. On continued incubation, threonine disappeared and was replaced by leucine (or isoleucine). The latter change paralleled in time the appearance of Fraction B, as observed previously (see Fig. 5), but was not correlated with the proportion of Fraction B observed chromatographically in this experiment. However, the progressive change in end groups is additional evidence for a sequence of reactions involving cleavage of peptide bonds. Separate experiments are being undertaken to study further the locus of the bonds involved in papain cleavage and the nature of the N-terminal groups of the purified 3.5 S Fractions A and B. In a similar study of a pathological macroglobulin, leucine (or isoleucine) but not threonine was released concurrently with the formation of 3.5 S fragments.

Action of Papain on Other Proteins—In exploratory study, it was found that new sedimenting components were not formed after either 2 or 4 hours of incubation of crystalline ovalbumin and crystalline human serum albumin with papain. A powdered sample of bovine serum albumin, presumably containing denatured protein, sedimented with a high meniscus indicative of peptides, but the major boundary had an s20,w of about 4 S. Several Bence-Jones proteins with an s20 = 3.6 S gave an intermediate boundary (1.5 S) that was diffuse. Although further study of the papain cleavage of Bence-Jones proteins might be worthwhile, specific cleavage to form stable fragments seems to be a phenomenon characteristic of γ-globulins.

The human globulins cleaved include not only normal γ-globulin and myeloma globulins of the 6.6 S size class (Table I) but also macroglobulins, i.e., pathological proteins of the high molecular weight 19 S class.11 As expected from the greater homogeneity of the myeloma globulins, the latter all yield more homogeneous 3.5 S fragments, as judged by starch gel electrophoresis. Abnormal globulins having mobilities higher than γ-globulin at pH 8.6 are readily cleaved. For individual myeloma proteins,

11 Stable 3.5 S fragments with the transient appearance of a 7 S intermediate are formed by papain cleavage of 19 S pathological macroglobulins. Several chromatographically and electrophoretically separable 3.5 S fractions have been obtained which appear to differ for individual macroglobulins. Tan, M. and Putnam, F. W., unpublished experiments.
several chromatographically separable 3.5 S fractions are obtained which are identifiable with the various electrophoretic components. However, the number and type of 3.5 S fragments vary for the individual specimens. Study is being made of the relationship to Fractions A and B of the 3.5 S fragments obtained from abnormal globulins.

DISCUSSION

Since Porter (10, 11) demonstrated the isolation of biologically active fragments from a papain digest of rabbit γ-globulin, many investigators have begun to apply this method to investigate the structure of antibodies and their combining sites and thus to study the nature of the antigen-antibody reaction. To validate this approach, one should investigate at least three problems: (a) the rate of formation of the fragments and the factors affecting this process, (b) the nature and stability of the fragments that are formed, and (c) the specificity of the cleavage and its locus. These problems have both general and practical significance. Any method permitting the production of reproducible stable fragments has obvious importance for the elucidation of protein structure. However, even if papain cleavage were limited to γ-globulins, the reaction deserves further investigation because the use of this approach to study the structure of antibodies will be brought into question unless the fragments are stable.

In regard to the first question, ultracentrifugal analysis of the papain cleavage products revealed that the initial cleavage of human γ-globulin occurs very rapidly, indeed, far more rapidly than one would expect from the incubation periods employed by Porter (10, 11) and subsequent workers (23). From the ultracentrifugal data, it was estimated that the initial rate of cleavage of γ-globulin was about as rapid as that for the most sensitive synthetic substrates for papain.

From these results, it is evident that prolonged incubation of γ-globulin antibodies with papain may be unwaranted as well as unnecessary, particularly for rabbit γ-globulin and for the pathological human proteins.

The limited nature of the cleavage reaction was surprising. Except for the single instance of porcine γ-globulin, no components intermediate between the 6.6 S protein and the 3.5 S fragments were detected. This was the case even when the reaction was stopped after 2 minutes of action of the activated enzyme or when unactivated mercuripapain was used for a long incubation period. Yet 5 S components were reported in earlier work with crude enzymes (4-6), and in a preliminary communication, Nisonoff et al. (29) have given evidence for a 6 S component when γ-globulin is exposed to the action of pepsin in the absence of reducing agents. At the other end of the size spectrum, ultracentrifugally resolvable components below 3.5 S were not observed, although a mixture of peptides was suggested by the fractionation with Sephadex and by the slowly sedimenting boundary at the meniscus of undialyzed preparations treated with crystalline papain. The absence of intermediate components and of a single characteristic cleavage peptide analogous to fibrinopeptide was also indicated by column chromatography and starch gel electrophoresis. It appears from these facts and from the ultracentrifugal homogeneity of the 3.5 S peak that the transition from 6.6 S globulin to 3.5 S fragments was both rapid and direct.

The factors affecting the cleavage reaction include the purity of the enzyme and the presence of activators or inhibitors. Crude papain used in earlier work (6, 7, 9) obviously must have given a mixture of fragments and many peptides. Crystalline papain is very effective in producing 3.5 S fragments but, presumably because of other protease impurities, also produces many random peptides. Mercuripapain is more specific, but the unactivated enzyme, though surprisingly effective, requires a long incubation period. The efficiency of unactivated mercuripapain probably depends on the liberation of sulfhydryl (or thiol ester) groups, either regenerated by dissociation of the mercury or by some kind of an exchange reaction. In this connection, it should be pointed out that concentrations of papain much lower than the 1:100 ratio used in this work are undoubtedly quite effective.

The nature of the fragments formed from human γ-globulin has so far not been identified in terms of biological activity, except that agar gel diffusion studies with anti-human γ-globulin rabbit serum showed that both Fractions A and B gave a precipitation line. However, the sample of Fraction A contained some 6.6 S protein. By analogy to rabbit γ-globulin and from the S0 value of 3.5 S, it may be assumed that three fragments of an approximate molecular weight of 50,000 are formed in the papain cleavage of human γ-globulin. Only two fractions have thus far been resolved chromatographically and electrophoretically, i.e. Fractions A and B. Fraction B appears to be formed from a precursor (not Fraction A) which is present in an early mixture of 3.5 S components that also contains Fraction A. In a number of instances in which Fractions A and B were separated by chromatography of the undialyzed digest, the ratio of A to B was almost exactly 2:1. This suggests that Fraction A may contain two similar (or identical) components analogous to Fractions I and II of rabbit γ-globulin, which contain the combining sites of the antibody. Correspondingly, Fraction B might be analogous to Fraction III, which has the chief antigenic determinants of the rabbit γ-globulin molecule. Unhappily for this argument, the sequence of chromatographic elution of I and II relative to III at pH 5.5 is the opposite of that of A versus B for human γ-globulin at pH 6.0. Moreover, in starch gel electrophoresis at pH 5.5, the sequence of mobilities of Fractions A and B of human γ-globulin is also the reverse of that expected if Fraction A were analogous to Fractions I and II of rabbit γ-globulin; i.e. although Fraction A moves somewhat more rapidly to the cathode than does the undigested human protein, and Fraction B moves more slowly, Fractions I and II move more slowly to

13 Kimmel and Smith (22) state that mercuripapain is inactive and that Versene (EDTA) alone produces no activation of papain for synthetic substrates. However, they found that at pH 4, mercuripapain dissociates to give a component devoid of mercury and a component with the structure HS—Papain—SH+. Other complexes of mercury and papain are possible, including hexamers of mercuripapain and a complex with an equimolar ratio of mercury and papain. It seems unlikely that mercuripapain could be activated by a sulfhydryl exchange reaction with γ-globulin, for the latter protein does not contain any free sulfhydryl groups in the native state. Karush, F., personal communication.

14 In all cases in which the ratio of Fraction A to Fraction B was found to be about 2.0, the separation of Fraction B was achieved by chromatography of the undialyzed digest in the presence of CMB-papain. In these cases, the peptides were separated from Fraction B either by later dialysis or by initial elution at a lower pH. However, when the whole digest with added CMB was dialyzed at length before stepwise elution of Fractions A and B, the proportion of Fraction B was decreased.
the cathode than does the undigested rabbit \( \gamma \)-globulin, and \( \gamma \) moves more rapidly.\(^\text{14}\) The order of chromatographic elution and of electrophoretic mobility, of course, depends on the net charge of the fragments, and there is no reason that functionally analogous fragments from rabbit and human \( \gamma \)-globulins should have the same charge.

Alternatively, it might be thought that since human \( \gamma \)-globulin is heterogeneous, many sets of 3.5 S components might be formed, each characteristic for a particular 6.6 S component. This suggestion fits with the broad zone for Fractions A and B on gel electrophoresis, but there are two arguments against it. In the first place, Fraction B is derived from already formed 3.5 S components rather than from residual, resistant 6.6 S molecules. Secondly, in a separate study\(^\text{14}\) it has been found that Fractions A and B were both formed (though not in the same ratios) when five subfractions of the immune \( \gamma \)-globulin from a single individual were incubated with papain. The subfractions had been prepared by electrophoresis-convection, and the antibody titer was sharply localized in several of the fractions. Also, if the \( N \)-terminal determinations merit significance, the cleavage points would appear to be similar in most of the globulin components of the pooled normal protein (and also in the pathological macroglobulin).

The stability of the 3.5 S fragments was put in question by our early results with rabbit \( \gamma \)-globulin, by the sequential appearance of Fractions A and B, and by the known broad specificity of papain. However, analysis of Fraction A purified from digests incubated for 16, 25, and 48 hours indicated that the amino acid composition was the same within experimental error. The chromatographic and electrophoretic properties of Fraction A likewise appeared to remain unchanged with time. \( N \)-Terminal group determinations corroborated the ultracentrifugal and chromatographic evidence that the 3.5 S material of Peak A obtained from short incubations does contain a component which changes on prolonged incubation. This component is presumed to be the 3.5 S precursor of Fraction B. With this reservation, Fraction A appeared to be remarkably resistant to further action by papain. The constancy with time of the amino acid composition of Fraction A indicated that the 3.5 S material was not progressively degraded by endwise loss of peptides to yield a series of related but increasingly smaller units. Rather it appeared that Fraction A was highly resistant to further action by papain, but if attacked, was degraded completely to dialyzable peptides. This conclusion accords with \( N \)-terminal group determinations and with the constancy of the physical properties of Fraction A.

Less evidence was obtained for the stability of Fraction B. The chromatographically separated material was more susceptible to further action by papain than was Fraction A. The ultracentrifugal boundary exhibited greater spreading than that for Fraction A, but the starch electrophoretic zone of B was sharper. Amino end group determinations on the dialyzed digests suggested that a series of related B fractions was not being formed. Although amino acid analysis of Fraction B obtained at two different times suggested that its composition varied, inadequate data were obtained to support this conclusion.

The specificity of the cleavage of human \( \gamma \)-globulin into 3.5 S components by papain seems established by (a) the discrete nature and relative stability of Fractions A and B, (b) the nearly stoichiometric appearance of new amino end groups, and (c) the similar cleavage of the \( \gamma \)-globulins of other species in contrast to the failure of other native proteins such as crystalline egg albumin and human serum albumin to undergo any change in ultracentrifugal properties after 4 hours of incubation with activated mercapto-papain. However, the locus of cleavage remains to be clarified. Semiquantitative determinations on the dialyzed papain digest indicate the presence of three major types of amino end groups. Of these, aspartic and glutamic acids are present in the original protein in the ratio of about 1:2, and were also found in the same ratio at several stages of incubation. Presumably, these amino acids represent the end groups of the original polypeptide chains, rather than cleavage points. However, the new end groups liberated, threonine in the 10-minute incubation and leucine (or isoleucine) on prolonged incubation, must represent a cleavage point containing a sensitive peptide bond. The new polypeptide chain was probably part of one of the original \( N \)-aspartyl or \( N \)-glutamyl chains. It may have been located in a region of the molecule where disulfide (or thiol ester) bonds stabilizing the configuration were buried. On papain cleavage of the peptide bond, the sulfur-containing bond perhaps became accessible to disruption by the cysteine present as an enzyme activator.\(^\text{15}\)

The specific bond broken in cleavage to 3.5 S units may be either peptide or thiol ester in nature. Although papain has broader specificity than trypsin, its greatest activity also is on substrates which have strongly cationic groups in the side chain, such as benzoyl-\( L \)-argininamide. Indeed the latter, although the most sensitive substrate known for the proteolytic action of both papain and trypsin, is more rapidly hydrolyzed by papain (22, 32-34). The sensitive peptide bond in human \( \gamma \)-globulin thus may involve the carboxyl group of arginine or lysine and the amino group of threonine or one of the leucines. Although \( L \)-arginyl-\( L \)-leucine is not hydrolyzed by papain, the carbobenzyloxynitro derivative is somewhat sensitive (35), yet it seems doubtful that the leucine amino end group liberated on prolonged incubation comes from such a peptide bond. Papain is also active on synthetic substrates in which glutamic acid, glutamine, or leucine provide either the carboxyl or the amino group of the peptide bond. Of the five bonds readily split by papain in the \( A \) chain of insulin, three involve combinations of two of the above amino acids (36). Although a bond such as Glu.Leu would appear susceptible, this is only slowly cleaved in insulin by pepsin (37), which also forms 3.5 S fragments of rabbit \( \gamma \)-globulin antibody in the presence of reducing agents (30). It must not be overlooked that papain has esterase activity (14) and also that at

\(^{14}\) Tan, M. and Putnam, F. W., unpublished experiments.
pH 5 (but not at pH 7.5) papain is able to cleave thiol esters in the absence of the usual activators and EDTA (38). Thiol esters have been postulated as existing at the active sites of enzymes such as papain and as covalent bonds involved in maintaining the three dimensional structure of proteins (39). Such a bond would have the feature of uniqueness necessary to explain the specific cleavage.

Whatever the locus of cleavage, there is no necessary relationship between its nature and the function and structure of γ-globulins or antibodies. Immunologically active fragments of these proteins have been obtained by digestion with pepsin (1-3), or trypsin (4, 5), as well as papain (which, to be sure, has some of the specificity of each of the other proteases). Yet thus far, proteolytic cleavage to yield reproducible protein subunits analogous to those formed from γ-globulins is a rare phenomenon. To be sure, trypsin (40) and the PR enzyme (41) produce half-globulins or antibodies. Immunologically active fragments of these molecules (42)), and zymogen activation by proteases is well known to involve the cleavage of specific peptide bonds. Further study of the locus (or loci) of cleavage now under way in our laboratory may add to knowledge of the structure of γ-globulins. It will be of interest to ascertain whether the sites of cleavage are the same for normal and pathological human globulins and for the globulins of animal species.

SUMMARY

Papain cleaves normal and pathological human γ-globulins into several chromatographically and electrophoretically separable 3.5 S fragments without the detectable formation of intermediates. The γ-globulins of various animal species are similarly cleaved, though at different rates. With the use of para-chloromercuribenzoate as an inhibitor, the rate of cleavage of normal pooled human γ-globulin and the products formed have been studied by chromatography, ultracentrifugation, starch gel electrophoresis, and analysis for amino acid composition and N-terminal groups. With activated mercuripapain, the initial reaction is rapid, yielding 80% 3.5 S fragments in 10 minutes plus a resistant 6.6 S fraction and dialyzable peptides. Unactivated mercuripapain produces slow cleavage but fewer peptides.

Two chromatographic components are formed (Fractions A and B). Fraction A predominates initially, and Fraction B appears progressively until their ratio approaches 2.0 in some experiments. Fraction A, isolated chromatographically after long incubation periods, is resistant to further enzyme action and is not converted to Fraction B. The amino acid composition of Fraction A is constant with time and resembles that of the intact γ globulin. During the cleavage, neither free amino acids nor a specific polypeptide is liberated; rather, a mixture of peptides results from random degradation of the 3.5 S fragments or the resistant 6.6 S protein. However, a new N-terminal amino acid, threonine, is liberated at early stages of incubation and is later replaced by leucine (or isoleucine). The specificity of the cleavage reaction is discussed and likewise its significance with regard to the structure of γ-globuline.

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The Cleavage of Human γ-Globulin by Papain
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