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Immunologically Active Fragments of Rabbit Gamma Globulin*

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Fragment III of papain-digested rabbit gamma globulin was digested with crystalline pepsin within a dialysis bag. A variety of degradation products resulted, some of which were too large to pass through the dialysis membrane. These retained the capacity to precipitate with antiserum against rabbit gamma globulin and demonstrated that a separation of antigenic determinants on the parent molecule had been effected by digestion. Other fragments were small enough to pass through the dialysis bag, did not precipitate with antibody, but were able to inhibit precipitation of antibody with fragment III. Fractionation by starch-gel electrophoresis and chromatography on Sephadex gels yielded a variety of fractions, some of which were able to combine with specific antibody, elicit reversed passive cutaneous anaphylaxis, and react with rheumatoid sera. One of the active fractions was comprised of peptides small enough to enter Sephadex G-50, indicating a molecular weight range of 2,000–10,000.

Rabbit γ-globulin possesses a number of properties of interest to immunologists in addition to its behavior as specific antibody. Degradation of the γ-globulin molecule by papain (Porter, 1959) yields a fragment (III) which, while devoid of antibody activity, carries the γ_2-globulin-specific determinants of the parent molecule (Porter, 1959), the ability to fix complement (Ishizaka *et al.*, 1962), the capacity to sensitize tissue for reversed passive cutaneous anaphylaxis (Ovary and Karush, 1961), reactivity with sera from cases of rheumatoid arthritis (Goodman, 1961), and the capacity to be recognized as γ-globulin by homologous cells (Brambell *et al.*, 1960).

Pepsin also splits γ-globulin into smaller units (Nisonoff *et al.*, 1960), but apparently further degrades the portion of the molecule corresponding to fragment III. This suggested the possibility that smaller pieces of fragment III bearing the above activities might be obtained by "controlled" treatment with pepsin. Accordingly, it was decided to digest fragment III within a dialysis sac under mild pressure, in order to protect peptides which were small enough to pass through the membrane from further degradation. An earlier report described preliminary results employing this procedure (Goodman, 1963).

MATERIALS AND METHODS

Rabbit γ-Globulin.—Rabbits were bled by cardiac puncture and the sera were pooled. γ-Globulin was prepared from the pooled sera by precipitation with sodium sulfate (Kekwick, 1940).

Antisera.—Goat antisera to rabbit γ-globulin and fragment III of papain-digested rabbit γ-globulin were kindly supplied by Dr. Melvin Cohn. The immunochemical characterization of these antisera has been described (Goodman and Gross, 1963). Another goat antiserum against rabbit γ-globulin provided by Dr. Leon S. Kind was used in passive cutaneous anaphylaxis experiments. From precipitin tests this antiserum was found to contain approximately 180 μg antibody N/ml.

Enzymatic Digestions.—Rabbit γ-globulin was digested by twice-crystallized mercuripapain (Worthington Biochemical Co.) and chromatographed on the cation-exchange resin carboxymethyl-cellulose (Carl Schleicher and Schull Co.), following the procedures described by Porter (1959). Three fractions were obtained, designated fragments I, II, and III in order of elution from the column. These were dialyzed exhaustively against water and lyophilized.

Fragment III of papain-digested γ-globulin was dissolved in water and the *p*H was adjusted to 4.0. The protein concentration was about 5%. Twice-crystallized pepsin (Mann Chemical Co.), dissolved in water at *p*H 4.0, was added at a concentration of 3% that of fragment III. The mixture was placed in a dialysis sac (Fisher Scientific Co.) which had been washed with running tap water for at least 12 hours. The dialysis bag was exposed to about 2 psi air pressure and placed in a vessel containing water at *p*H 4.0 at room temperature. The dialysate, about 50 ml, was changed twice daily for a period of 4 days. During this pressure dialysis the volume within the bag decreased and a crystalline precipitate formed. At the end of the fourth day both the dialysate and the material remaining within the bag were adjusted to *p*H 7.5 and the dialysate was lyophilized.

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Analytical Methods

Protein Determinations.—Quantitative analyses of γ-globulin and fragment III were performed spectrophotometrically at 277 mμ in 0.25 N acetic acid (Goodman and Gross, 1963). Digestion products of fragment III, for which extinction coefficients at this wavelength were unknown, were determined using a modified micro-Kjeldahl technique (Markham, 1942). Eluates from Sephadex columns were analyzed using ninhydrin (Rosen, 1957).

Starch-Gel Electrophoresis.—This technique was employed as described by Smithies (1955), with 0.2 M sodium borate buffer at *p*H 8.4. A potential difference of 250 volts was applied for 15 hours at room temperature. For preparative purposes, the bands of protein were located by staining a thin slice of the gel. Sections of gel corresponding to positions of the bands were cut out and minced, and the proteins were eluted from them by electrodialysis (Moretti *et al.*, 1958).

Chromatography on Sephadex Gels.—The dialyzable portion of pepsin-digested fragment III was fractionated by passage through Sephadex G-50 and Sephadex G-25 (Porath, 1960). The column size used for G-50 was 3.0 × 45 cm and a column with dimensions of 1.4 × 55 cm was used for G-25. The solvent in both cases was 0.01 M NaCl. Fractions comprised of peptides large enough to be excluded from G-25 were desalted by passage through this gel using water as the eluent.

Fingerprinting.—The peptides in one of the fractions resulting from chromatography of the dialysate on Sephadex gels were separated by two-dimensional electrophoresis chromatography (Ingram, 1958). About 1.0 mg of protein was applied to a small area of a sheet of Whatman No. 1 filter paper. The peptides were subjected to electrophoresis in one dimension at a potential difference of 2000 v for 45 minutes. The solvent was pyridine–acetic acid–water (10:0.4:90), *p*H 6.4. The paper was then thoroughly dried and subsequently exposed to descending chromatography in 1-butanol–acetic acid–water (120:30:50). Peptides were detected by spraying the paper with ninhydrin (0.25% in acetone) and heating it at 70° for 15 minutes, followed by incubation in the dark at room temperature for several days.

Ultracentrifugation.—Fragment III and fractions of pepsin-digested fragment III were examined in a Spinco Model E analytical ultracentrifuge. The solvent was 0.1 M NaCl and a speed of 59,780 rpm was employed. Protein concentrations were 10 mg/ml.

Immunologic Assays

Gel Diffusion and Immunoelectrophoresis.—The methods described by Ouchterlony (1953) and Scheidegger (1955) were applied to the analysis of digested fragment III. Concentrations of antigen ranged from 0.1 to 5% and the antiserum employed was the goat anti-rabbit γ-globulin obtained from Dr. Melvin Cohn. Immunoelectrophoretic analyses were run in sodium barbital buffer, *p*H 8.6, at a potential difference of 400 v for 1 hour.

Delay in Flocculation of Antigen-Antibody Reactions.—Digestion products of fragment III which did not give detectable precipitin reactions in agar with anti-γ-globulin serum were assayed for ability to combine with antibody by their capacity to delay flocculation of a goat anti-fragment III serum with its homologous antigen. Aliquots (0.5 ml) of serum, containing about 34 μg antibody N, were incubated with the fractions being assayed in an electrically heated dry incubation rack at 37° for about 15 minutes. Sixteen μg of fragment III, an amount calculated to be just on the antibody excess side of the equivalence region of the precipitin curve for this serum (Goodman and Gross, 1963), was then added to each tube, including a control containing saline instead of inhibitor. The time required for flocculation to occur was noted for each tube. Periods of more than double that required for flocculation of the control were considered evidence of a significant degree of reaction between inhibitor and antibody. The flocculation time for the control in various experiments was 50–60 minutes. Inhibitors were tested simultaneously in an unrelated antigen-antibody system (bovine serum albumin and rabbit anti–bovine serum albumin). None of the fractions showed any capacity to significantly delay the flocculation of that system.

Passive Cutaneous Anaphylaxis.—The capacity of products of degraded fragment III to fix to animal tissue was determined by inhibition of the passive cutaneous anaphylaxis reaction (Ovary, 1958). The abdomens of guinea pigs weighing about 350 g were shaved 24 hours prior to experiments. The animals were injected intracutaneously with 0.1-ml volumes containing 0.3 μg of rabbit anti–bovine serum albumin antibody plus 200 μg of either fragment III or one of the fractions from its digestion by pepsin. A total of four injection sites were used on each guinea pig. Two of these were controls with antibody plus fragment III and antibody plus saline. Three hours later the guinea pigs were injected intravenously with 15 mg of bovine serum albumin in 0.5 ml of Evans' blue dye. After another period of 30 minutes the skins were retracted and the presence or absence of dye in the injected area was noted. Fragment III inhibited passive cutaneous anaphylaxis in all cases whereas the saline control areas showed uniformly positive reactions.

The ability of products of fragment III to elicit reversed passive cutaneous anaphylaxis in guinea pigs (Ovary, 1958) was determined by intracutaneous injection of 0.1-ml volumes of the various protein fractions. Each pig was injected in one of two sites with 0.2 μg of fragment III or 20 μg of fragment I of papain-digested γ-globulin to serve as positive and negative controls, respectively. The peptic fractions of fragment III were assayed at 20-μg, 2-μg, and 0.2-μg levels. Three hours after the intracutaneous injections the animals were inoculated intravenously with 1.0 ml of a mixture containing equal volumes of Evans' blue dye and goat anti–rabbit γ-globulin serum. Following a 30-minute interval the guinea pig skins were examined for the presence of dye in the injected areas. The fragment III controls were always positive while the fragment I controls were uniformly negative.

Reactivity with Rheumatoid Sera.—The capacity of fractions of pepsin-digested fragment III to react with sera from rheumatoid arthritics was determined as described previously (Goodman, 1961). Fragment III (3.5 mg) and the peptic fractions were each dissolved in 2.0 ml of *p*H 8.0 buffered saline and absorbed overnight at 4° with 2.0 ml of washed, packed sheep erythrocytes from 2-day-old blood. These absorbed protein solutions were used to coat tanned sheep erythrocytes. The coated cells were subsequently assayed for agglutinability by a variety of rheumatoid sera provided by Dr. Wallace V. Epstein. Controls included tanned uncoated cells plus serum and coated cells plus saline. These were not agglutinated by the rheumatoid sera in any of the tests.

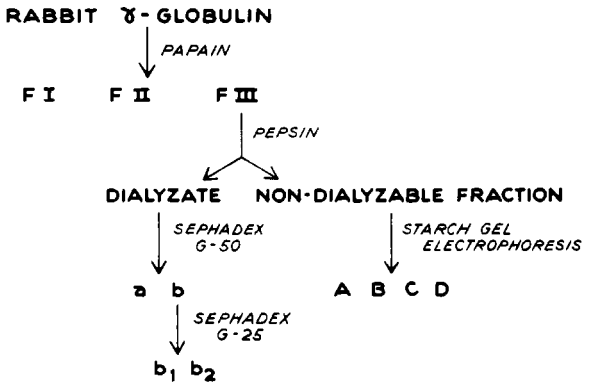

FIG. 1.—Flow diagram of digestion and fractionation of digestion products of rabbit γ-globulin.

RESULTS

A flow diagram of the digestion and fractionation of rabbit γ-globulin is shown in Figure 1. About 540 mg of fragment III from papain-digested γ-globulin was treated in a dialysis bag with 3% crystalline pepsin. Approximately 75% of the protein appeared in the dialysate after 4 days. The rest remained within the bag, some as a crystalline precipitate which formed during the course of the digestion. The optical density at 280 mμ of the dialysate at the time that digestion was terminated indicated that very little, if any, protein was still diffusing across the membrane.

The material left inside the dialysis bag, referred to as the nondialyzable fraction, the dialysate, fragment III, and γ-globulin were electrophoresed in starch gel; the patterns which were obtained are shown in Figure 2. Solutions of 50 mg/ml were used in order to detect components which might be present at low concentration. This concentration far exceeded the solubilities of fragment III and the nondialyzable fraction. γ-Globulin gave a broad smear under the conditions of electrophoresis employed and no banding was seen. Fragment III displayed four distinct bands, the two nearer the origin being much the heavier, and a considerable amount of protein which remained at the origin. The nondialyzable fraction gave four bands which were identical in position to those of fragment III, but their quantitative distribution differed in that more protein was present in the faster bands and little remained at the origin. The dialysate showed only fast-moving components, three of which agreed in position with bands given by fragment III and the nondialyzable fraction; the remainder appeared as a diffuse smear near the anode end of the gel.

The four bands of the nondialyzable fraction, designated A, B, C, and D in order of increasing distance from the gel origin, were isolated by elution from starch. Each was examined in an analytical ultracentrifuge and displayed a single, symmetrical peak with the following sedimentation coefficients: A, 2.3 S; B, 3.2 S; C, 2.6 S; D, 2.5 S. Since fragment III has a sedimentation coefficient of about 3.6 S, these figures suggest a smaller molecular size for A, C, and D, while B would appear to be very similar in size to the parent molecule. The dialysate did not show a peak which moved away from the meniscus in the ultracentrifuge.

Whereas fragment III gave a single band of precipitate with goat anti-rabbit γ-globulin serum in agar diffusion tests, the nondialyzable fraction showed four bands (Fig. 3). Components A, B, and C each gave a band which fused with the band given by fragment III. Spurs on the fragment III bands,

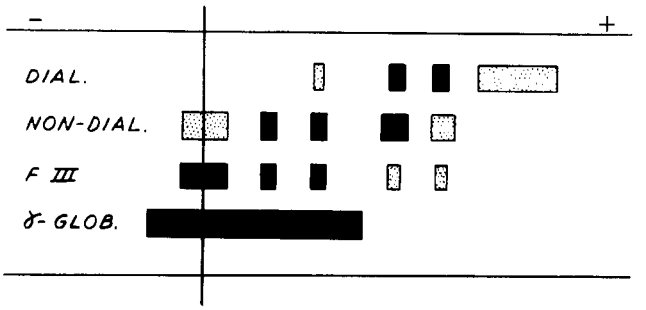

FIG. 2.*—Electrophoretic patterns in starch gel of dialysate (DIAL), nondialyzable fraction (NON-DIAL), fragment III (FIII), and rabbit γ-globulin (γ-GLOB). The solid areas indicate heavy protein concentrations; the speckled areas indicate lower concentrations. * Appeared in *Science 139*, 1292 (1963).

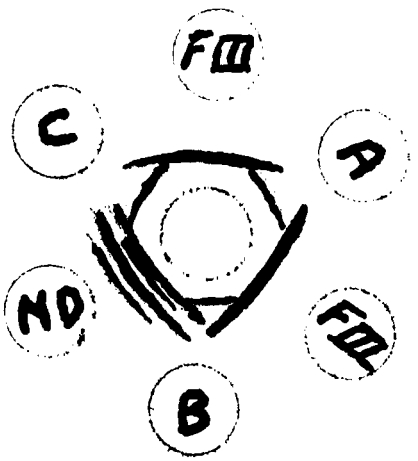

FIG. 3.—Gel-diffusion patterns of fragments of rabbit γ-globulin reacted with goat anti-rabbit γ-globulin. Goat antiserum is in central well. Fragment III (FIII), nondialyzable fraction (ND), and components A, B, and C from starch-gel electrophoresis of the nondialyzable fraction are in the peripheral wells.

however, indicate that the components are antigenically deficient relative to fragment III. The dialysate in this experiment did not give detectable precipitin bands at a concentration of 10 mg/ml. However, other digestions performed under essentially identical conditions yielded dialysates which precipitated with the antiserum.

Immunoelectrophoresis gave clearer evidence of the cleavage of fragment III (Fig. 4). Fragment III gave a single precipitin arc which, however, was not symmetrical, while the nondialyzable fraction gave five distinct lines which ranged in position from the cathode to the anode end of the antiserum trough. Each of the four components of the nondialyzable fraction gave a single arc with a characteristic electrophoretic mobility. Fraction A was the most basic of the four, its position being closest to the cathode, while fraction D migrated most rapidly toward the anode. This agreed with their relative positions in starch-gel electrophoresis. When the four components were compared by the Ouchterlony double-diffusion technique, they gave bands of complete identity with each other (Fig. 5), indicating, insofar as this antiserum could discriminate, that they all carried identical antigenic determinants.

The dialysate was fractionated by molecular sieving on Sephadex gels. Passage through Sephadex G-50, which has an exclusion weight of about 10,000 for dextrans (Porath, 1960), yielded two peaks, designated

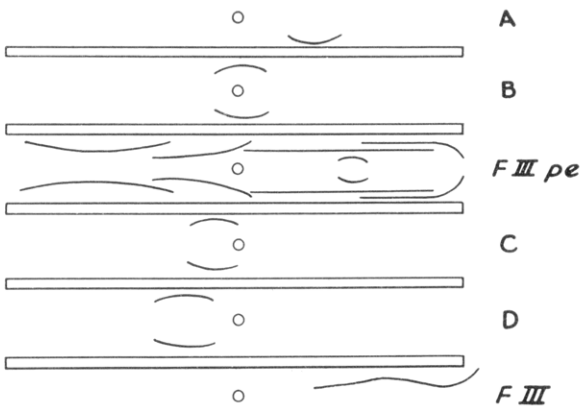

FIG. 4.*—Immunoelectrophoretic patterns of fraction III (FIII) and its digestion products. FIII pe is the nondialyzable fraction from peptic digestion of FIII; A, B, C, and D are the protein bands from starch-gel electrophoresis of FIII pe. Protein concentrations are 10 mg/ml. The troughs contain goat anti-rabbit γ-globulin serum. The cathode is at the right. * Appeared in *Science 139*, 1292 (1963).

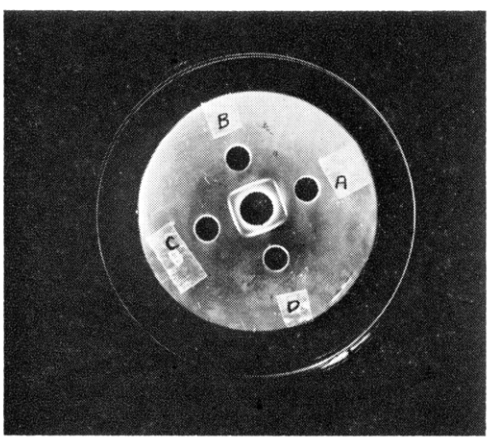

FIG. 5.—Gel-diffusion patterns of components of nondialyzable fraction of pepsin-digested fragment III recovered from starch gel. Goat anti-rabbit γ-globulin is in central well. Concentration of components in peripheral wells is 2 mg protein/ml.

(a) and (b) in order of elution from the column (Fig. 6). It was established that fraction (a) consisted of molecules too large to enter the gel by determining the void volume of the column using bovine serum albumin. Fraction (a) was eluted with precisely the volume of effluent required to remove the albumin. Fraction (b) consisted of molecules which were small enough to enter the gel and was very broad, indicating a considerable degree of heterogeneity in molecular size within the fraction. Protein, as determined by optical density at 280 mμ, did not coincide with values given by ninhydrin analysis (Fig. 6); fractions were cut on the basis of ninhydrin values. Thus, fraction (a) consisted of the effluent from 50–90 ml while fraction (b) comprised the effluent from 100 ml to the point at which no more protein could be detected.

Fraction (b) was sieved through Sephadex G-25, which has an exclusion weight of 2000–3000 (Porath, 1960), and two peaks resulted, their breadth again suggesting considerable heterogeneity with respect to molecular size (Figure 7). The peaks were termed (b_1) and (b_2) in order of elution from the column. Fraction (b_1) began to appear immediately after passage of the void volume but tailed markedly. The peaks were divided at the 50-ml effluent point.

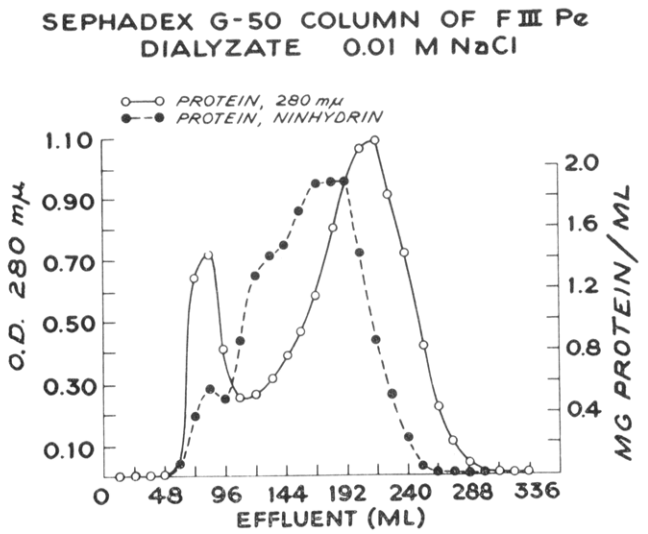

FIG. 6.—Dialysate from pepsin-digested fragment III chromatographed on Sephadex G-50.

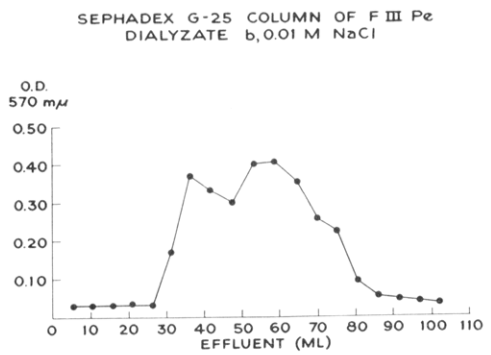

FIG. 7.—Fraction b, the material which entered Sephadex G-50, chromatographed on Sephadex G-25. Protein determined by reaction with ninhydrin.

None of the fractions of the dialysate formed discernible precipitin arcs with goat anti-γ-globulin in immunoelectrophoresis when tested over a protein concentration range of 1.0–50 mg/ml. However, fractions (a) and (b_1) were capable of delaying the flocculation of antibody with fragment III. One-half mg of (a) or (b_1) caused a delay of several days in flocculation. Since flocculation occurred within about 50 minutes in the absence of these inhibitors, the degree of inhibition was very pronounced. This reaction was specific since the flocculation of an unrelated antigen-antibody system was unaffected by either (a) or (b_1). The same amount of fraction (b_2) was ineffective in inhibiting the homologous system and thus (b_2) did not appear capable of combining with antibody.

None of the fractions of the nondialyzable fraction or of the dialysate inhibited passive cutaneous anaphylaxis in guinea pigs when 0.2 mg of protein was used. The same quantity of fragment III produced complete inhibition. On the other hand, reversed passive cutaneous anaphylaxis was elicited by 20 μg of fractions A, B, C, and (b_1) (Table I). Fraction D was not tested due to inadequate supply; fractions (a) and (b_2) gave negative results. Only 0.2 μg of fragment III was necessary for a positive reaction, whereas all fractions tested were negative or questionable at 2-μg levels. Therefore it would seem that fragment III is 10- to 100-fold more effective on a weight basis than any of its split products in eliciting reversed passive cutaneous anaphylaxis.

TABLE I
REVERSED PASSIVE CUTANEOUS ANAPHYLAXIS IN GUINEA PIGS

Antigen	Quantity of Antigen (μg) 0.2	2	20
Fragment I			−
Fragment III	+		
Fraction A	−	−	+
Fraction B	−	−	+
Fraction C	−	−	+
Fraction a			−
Fraction b_1	−	−	+
Fraction b_2			−

TABLE II
AGGLUTINATION OF SHEEP ERYTHROCYTES COATED WITH PROTEIN FRACTIONS BY RHEUMATOID SERA

Serum	Protein Coat (titer) Fragment III	Fraction A	Fraction B	Fraction C	Fraction b
Goat anti-fragment III	32768	32768	32768	16384	512
Rheumatoid pool	224	0	28	0	112
Individual rheumatoid sera					
1	112	0	0	0	0
2	7168	28	112	0	0
3	112	0	28	0	0
4	56	14	56	28	14
5	112	0	28	0	0
6	112	0	7	0	0

Data on the reactivity of rheumatoid sera with sheep erythrocytes coated with fragment III and some of the fractions are tabulated in Table II. All cells were strongly agglutinated by goat anti-fragment III serum, demonstrating the presence of the protein coats. The titer of the serum with cells coated with fraction (b) was only 1:512, considerably lower than the titers of 1:16,000–1:32,000 obtained with the other coated cells. A pool of ten rheumatoid sera as well as six individual sera were tested for their ability to agglutinate the coated cells. The rheumatoid pool reacted similarly with fraction (b)–coated cells and fragment III–coated cells, the titers for the two being 1:112 and 1:224, respectively. However, only one of the six individual sera agglutinated fraction (b)–coated cells. Cells coated with fraction B were agglutinated almost as consistently as were cells coated with fragment III. Only one serum failed to react with these cells; this serum failed to agglutinate any of the cells coated with fractions of pepsin-digested fragment III although it gave a titer of 1:112 with fragment III–coated cells. Only one serum reacted with fraction C cells and this serum agglutinated every type of coated cell tested.

Fraction (b_1), which contained peptides small enough to enter Sephadex G-50 but too large to enter G-25, was analyzed by two-dimensional electrophoresis-chromatography and found to consist of a complex array of components. In addition to a smear of protein remaining near the origin, about fifty spots developed on the paper. The major components appeared to be nearly isoelectric at the *p*H of the electrophoretic run (*p*H 6.5). Since most of the peptides migrated rapidly with the solvent used for descending chromatography, an attempt was made to separate these peptides from the material remaining near the origin by passage of (b_1) through a small cellulose column using the same eluent (1-butanol–acetic acid–water). However, when the effluent was tested by fingerprinting it was found to contain essentially the original mixture of peptides, including the protein which remained near the origin.

DISCUSSION

When fragment III of papain-digested rabbit γ-globulin is treated with pepsin, a variety of smaller peptides is produced. Some of these, representing no more than about 25% of the starting material, are still relatively large molecules with sedimentation coefficients ranging from 2.3 S to 3.2 S and retain the capacity to precipitate with antiserum against rabbit γ-globulin. Others are small enough to pass through dialysis tubing and can enter Sephadex G-50, placing them within molecular weight limits of approximately 2,000–10,000. Some of these smaller peptides can combine with antibody, though unable to form precipitates, as evidenced by their ability to delay flocculation of antibody with fragment III. Peptides small enough to enter Sephadex G-25, which have molecular weights of less than about 2000, showed no evidence of ability to combine with antibody since as much as 0.5 mg of protein was unable to significantly delay flocculation of 34 μg of antibody N by 16 μg of fragment III.

When the constituents of the nondialyzable fraction, as resolved by starch-gel electrophoresis, were tested individually by double diffusion (Fig. 5), each gave a single band of precipitate and reactions of identity with the other components, indicating that they all carried identical antigenic determinants. Immunoelectrophoretically, each gave a short precipitin arc with a characteristic mobility, but together they covered only a small portion of the range of precipitin lines shown by unfractionated nondialyzable fraction (Fig. 4). This indicates that much of the nondialyzable fraction was not recovered from starch. Since a considerable amount of protein remained at the origin of the starch gel (Fig. 2), the unrecovered components may reside there. The nondialyzable fraction is presently being fractionated by other methods in an attempt to clarify this point.

The coincident positions of the protein bands on starch given by fragment III and the nondialyzable fraction would make a physicochemical and immunologic comparison of these bands of interest. Unfortunately, the very low solubility of fragment III at *p*H values near neutrality precludes the possibility of recovering the necessary quantities of its components from starch gels. We are currently fractionating both fragment III and the nondialyzable fraction by ion-exchange chromatography on a scale sufficiently large to permit studies of this kind.

The dialysate did not form precipitin bands in agar when tested at concentrations up to 50 mg/ml. However, in some other peptic digestions of fragment III, under conditions manifestly identical to those described here, the dialysate did contain precipitating fragments. This inconsistency was probably due to alteration of the dialysis tubing under pressure. Although care was taken to maintain the pressure at 2 psi in each experiment, slight variations might have caused undue "stretching" of the membrane at times, accounting for a change in its porosity. It seems reasonable that the dialysate always contains a mixture of precipitating and nonprecipitating peptides and that their relative concentrations determine whether or not precipitation will occur. The precipitating peptides would be expected to appear in fraction (a), which consisted of molecules too large to enter Sephadex G-50. How-

ever, fraction (a) did not show precipitin lines with the goat antiserum either, and inhibitors may also have been present in this fraction in relatively high concentration.

Three components of the nondialyzable fraction as well as fraction (b_1) of the dialysate showed activity in eliciting reversed passive cutaneous anaphylaxis in guinea pigs (Table I) and reactivity with human rheumatoid sera (Table II). However, these materials were usually significantly less active than undegraded fragment III. This raises the possibility that contamination by a small quantity of undegraded fragment III might be responsible for the activities of the fractions. While it is impossible to rule out contamination of the components of the nondialyzable fraction, it would appear that such an explanation for the activity of fraction (b_1) is improbable. Twenty μg of fraction (a), consisting of molecules with molecular weights larger than about 10,000, failed to elicit reversed passive cutaneous anaphylaxis while the same quantity of (b_1) gave a strongly positive reaction. If contamination by fragment III were responsible for the activity, then fraction (a) should have been much more active than fraction (b_1), since fragment III, with a molecular weight of about 80,000, is too large to enter G-50 and most of it would have appeared in fraction (a). Unfortunately, there was too little of fraction (a) to test for activity with rheumatoid sera. However, the evidence clearly indicates that relatively small immunologically active peptides are produced by peptic digestion of fragment III.

The experimental data leave open the question of how many active peptides are produced, how large they are and whether the different activities involved are carried by the same or different peptides. The fingerprinting of fraction (b_1) revealed a great complexity of peptides. If only one or several of these peptides are responsible for the immunologic activities, then they could have very high specific activity since they would comprise only a small fraction of the total protein. The attempt to separate the fast-moving peptides from the protein remaining near the origin of the fingerprint by chromatographing fraction (b_1) on a cellulose column was unsuccessful since a fingerprint of the eluted protein, which came off the column as a single peak, showed essentially the same pattern of spots given by (b_1) before chromatography, including the protein remaining near the origin. For some reason this protein moved as rapidly as the other peptides on the cellulose column.

The pattern of reactivity of the various fractions with rheumatoid sera (Table II) suggests the existence of heterogeneity in the specificity of these sera for determinants on rabbit γ-globulin. Fraction B, with a sedimentation coefficient close to that of fragment III (3.2 S and 3.6 S, respectively), showed some reactivity with all but one of the seven sera tested. However, its activity with the rheumatoid pool was low (titer 1:28) while the activity of fraction (b_1) with this pool was appreciably greater (titer 1:112). On the other hand, (b_1) reacted with only one of the six individual sera, giving a lower titer than that obtained with fraction B–coated cells. Fraction A reacted with only two of the sera and fraction C with but one. The one serum that agglutinated all types of coated cells had a titer of only 1:56 with fragment III cells but appeared to be specific for determinants present in all the fractions. These results suggest that differences in specificity between the rheumatoid sera exist and these may be related to differences in specificity of rheumatoid sera for human γ-globulin. However, titers of most of the sera used in these investigations with rabbit γ-globulin were low and more extensive experimental work is needed before definite conclusions are warranted.

The specificity of individual rheumatoid sera has been shown to be directed against one or another of several genetically controlled determinants of human γ-globulin (Grubb, 1956; Fudenberg and Kunkel, 1961). The rheumatoid factors behave like isoantibodies and their cross-reactivity with rabbit γ-globulin is presumably due to structural similarities between determinants on the two kinds of molecules. Validation of this assumption must await isolation and characterization of the determinants involved.

Different genetic types of rabbit γ-globulin have also been found; these have been designated "allotypes" (Oudin, 1956). Isoantibodies against allotypic types of rabbit γ-globulin are thus analogous in some respects to rheumatoid factors in humans. However, the specificity of rheumatoid factors for rabbit γ-globulin is directed against determinants located almost exclusively on fragment III of the papain-digested molecule (Goodman, 1961), whereas allotypic antibodies appear to be specific for determinants found primarily on fragments I and II (Kelus *et al.*, 1960; Dubiski *et al.*, 1961; Leskowitz, 1963).

Other serum factors resembling human rheumatoid factors have been induced in rabbits by prolonged immunization with bacterial antigens (Milgrom and Witebsky, 1960; Abruzzo and Christian, 1961) and with soluble antigens (Williams and Kunkel, 1963). These substances are associated with the macroglobulin fraction of serum proteins and possess the properties of antibodies specific for autologous γ-globulin but with cross-reactivity against human γ-globulin. The agent responsible for eliciting the formation of these factors is believed to be autologous immune globulin altered by prolonged interaction with antigen *in vivo*. It has recently been reported that the reactivity of rabbit γ-globulin with these "rheumatoid factors" is diminished or abolished by treatment with pepsin (Christian, 1963), suggesting that their specificity is directed against determinants on fragment III. This is in marked contrast to the specificity of allotypic antisera but similar to that of human rheumatoid factors. A comparison of the structural units on fragment III of rabbit γ-globulin involved in reaction with human and with rabbit "rheumatoid factors" would clearly be of great interest.

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Dissociation of Rabbit γ-Globulin into Half-Molecules after Reduction of One Labile Disulfide Bond*

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Rabbit γ-globulin has previously been found to dissociate into half-molecules at low *p*H after reduction. The present investigation indicates that one-half to two-thirds of the molecules can be dissociated after reduction of one very labile disulfide bond. Dissociation of the remainder of the γ-globulin requires the reduction of more than one disulfide bond, indicating heterogeneity with respect to the number of interchain disulfide bonds, or to the relative lability of a single bond joining the half-molecules. The evidence is consistent with the conclusion that the bond joins two "A" chains. The amino acid composition of isolated half-molecules is consistent with other evidence indicating that each consists of an "A" and a "B" chain.

Recent investigations indicate that 6.5 S γ-globulins of several species consist of four polypeptide chains, two of approximate molecular weight 55,000 and two of molecular weight 20,000 (Edelman, 1959; Edelman and Poulik, 1961; Fleischman *et al.*, 1962, 1963; Pain, 1963; Marler and Tanford, 1963). The two types of polypeptide chain have been referred to as heavy ("H") and light ("L") chains (Edelman and Benacerraf, 1962), or as "A" and "B" chains (Porter, 1962), respectively. The combining sites of the molecule appear to be associated with "A" chains (Fleischman *et al.*, 1963; Utsumi and Karush, 1963); the participation of "B" chains, in a manner that is not as yet clearly defined, is also indicated (Edelman *et al.*, 1961, 1963; Franek and Nezlin, 1963; Roholt *et al.*, 1963; Metzger and Singer, 1963).

It was recently reported (Palmer *et al.*, 1963) that rabbit γ-globulin is cleaved into two subunits, approximately equal in size, by reduction with 0.1 M 2-mercaptoethylamine hydrochloride and subsequent acidification to *p*H 2.5 in 0.1 M NaCl. The conclusion that each subunit contains an A and a B chain was supported by the average molecular weight of the subunits; the agreement between the weight and *z*-average molecular weights, indicating homogeneity; the fact that the subunits migrate in the ultracentrifuge as a single symmetrical peak; and the low yield of B chains, obtained by gel-filtration on Sephadex in 1 M propionic acid, from a preparation which dissociated almost completely into half-molecules at low *p*H. Also, the present report will indicate that the half- and whole molecules are very similar in amino acid composition.

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After neutralization of a reduced, acidified preparation, the major product had a sedimentation coefficient almost identical with that of untreated (or reduced) γ-globulin, indicating that recombination of half-molecules occurs at neutral *p*H (Palmer *et al.*, 1963). Most of the specific combining capacity of an anti-hapten antibody is retained during these procedures, and the protein remains soluble. Subunits derived from specifically purified antiovalbumin are capable of combining with subunits of normal γ-globulin to form hybrid 6.2 S molecules with active combining sites (Nisonoff and Palmer, 1964).

Data presented here indicate that the dissociation of a large proportion of γ-globulin molecules at low *p*H occurs after the reduction of one disulfide bond per molecule. The bond is extremely labile to reduction by 2-mercaptoethylamine HCl. The results suggest that the disulfide bond links two A chains.

Materials and Methods

Three preparations of normal rabbit γ-globulin were used; each was obtained from the serum of an individual rabbit. The γ-globulin was prepared by three precipitations with decreasing concentrations of sodium sulfate (Kekwick, 1940) followed by passage through a column of diethylaminoethyl-cellulose (Levy and Sober, 1960) in 0.0175 M sodium phosphate buffer, *p*H 6.9. The $s_{20,w}$ values of the preparations were 6.2 or 6.3 S. Other serum proteins were not detectable by immunoelectrophoresis with sheep anti-rabbit serum.

S-Carboxymethyl-L-cysteine was obtained from the Nutritional Biochemicals Corp. 2-Mercaptoethylamine hydrochloride and sodium *p*-mercuribenzoate were obtained from the California Corp. for Biochemical Research.