Isolation and Fragmentation of Antibodies to Polytyrosyl Gelatin*

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Received October 21, 1963

Antibodies to polytyrosyl gelatin were isolated immunospecifically by the digestion of the antigen-antibody precipitate with collagenase. Pieces of the antigen which remained bound to the antibodies and blocked their activity were removed by passing the antibody preparation through a Sephadex column at acid *p*H. The antibodies thus isolated were 6.5 S γ-globulin and precipitated with added fresh antigen. By using radioactively labeled antigen it was possible to follow the antibody-combining sites by means of the pieces of the antigen which remained bound to the antibody before the passage through Sephadex. These antigen pieces were associated with fragments I and II of the antibody after fragmentation with papain, while fragment III was devoid of such pieces. After reduction with mercaptoethanol in aqueous solution and the separation of the subunits A and B of the antibody no radioactive pieces of the antigen were found in fraction B. The protein in fraction A, on the other hand, was associated with antigen pieces. Several alternative explanations for the presence of the antigen pieces in this fraction are discussed.

Immunological studies on gelatin modified by attachment of polypeptide chains have shown that polytyrosyl gelatin is a potent antigen in rabbits (Sela and Arnon, 1960). The serological specificity of various samples of tyrosylated gelatin depends on their tyrosine content. While a limited enrichment of gelatin with tyrosine enhanced the production of antibodies cross-reacting with gelatin, attachment of 10% of tyrosine residues to gelatin resulted in derivatives which elicited in rabbits antibodies directed exclusively toward the tyrosine peptides attached (Arnon and Sela, 1960a).

Antibodies to gelatin may be isolated, in the neutral *p*H range, by digestion of the gelatin-antigelatin complex with collagenase and removal of the degradation products of gelatin by dialysis (Arnon and Sela, 1960b). The pure antibody may be precipitated anew by the antigen. However, antibodies to a tyrosylated gelatin isolated in a similar way could not be precipitated by the homologous antigen. It was suggested that in this case the active sites of the antibodies remained blocked by pieces of the antigen. In this paper evidence is presented that upon purification by means of collagenase of antibodies to polytyrosyl gelatins possessing about 15% tyrosine residues, pieces of the antigen indeed remained bound to the active sites of the antibodies. The removal of these pieces upon passage through a Sephadex column under acid conditions yielded pure antibodies that could be precipitated anew by the homologous antigens.

Using radioactive polytyrosyl gelatin preparations, it should be possible to follow the antibody-combining sites by means of tagged antigen pieces bound to them. Thus, after the splitting of the antibodies to polytyrosyl gelatin with water-insoluble papain (Cebra *et al.*, 1961), tagged antigen pieces were found to be associated only with fragments known to possess the antibody-combining sites (Porter, 1959). The presence or absence of antibody-combining sites in the fragments obtained by the reduction of antibodies in aqueous solution (Fleischman *et al.*, 1962) was investigated in a similar way. The results suggest that fraction B (Fleischman *et al.*, 1962) does not contain active sites of the antibody, as essentially no radioactive pieces of polytyrosyl gelatin were found in this fraction.

* This investigation was supported in part by a U. S. Public Health Service research grant (AI-04715) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

EXPERIMENTAL

Poly-L-tyrosyl Gelatin (pTyrGel).[1]—This was prepared by reacting *N*-carboxy-L-tyrosine anhydride (Berger *et al.*, 1958) with gelatin (U.S.P. granular, Fisher Scientific Co.) in a way analogous to that described by Arnon and Sela (1960a). Approximately 0.3 g of the anhydride was used per 1 g gelatin. Labeled pTyrGel was prepared by reacting the gelatin with the *N*-carboxyanhydride of ^{14}C-labeled tyrosine (Radiochemical Center, Amersham, Engl.) or by iodination of pTyrGel with ^{131}I (50 μc/100 mg) according to Talmage *et al.* (1954), followed by exhaustive dialysis. The tyrosine residue content in the various preparations was calculated from the extinction of an alkaline solution (*p*H 13) at 293.5 mμ and found to be between 14 and 16%. The ^{14}C-labeled pTyrGel contained 14% tyrosine residues. The radioactivity of the ^{14}C-labeled pTyrGel was 12,500 cpm/mg and that of the ^{131}I-labeled pTyrGel was in the range of 100,000–500,000 cpm/mg at the beginning of the experiment.

Digestion of Immunological Precipitates by Collagenase.—Precipitates formed by pTyrGel and anti-pTyrGel rabbit serum were washed three times with cold 0.14 M NaCl and suspended in 0.05 M Tris buffer, *p*H 7.4, containing 0.005 M $CaCl_2$. Collagenase (95 units/mg, a gift from Dr. P. M. Gallop) was added to the suspension in an amount calculated to be approximately 1 unit/mg pTyrGel (Seifter *et al.*, 1959). After 3 hours at 37° most of the precipitate was dissolved, while no such effect was found when immunological precipitates of ovalbumin or bovine serum albumin were treated similarly. The small amount of insoluble material was removed by centrifugation and the antibodies were separated from the free degradation products of the antigen by passing the supernatant through a Sephadex G-100 (Pharmacia, Uppsala, Sweden) column at neutral *p*H. Another way of removing the free degradation products of the antigen was by precipitation at 0.37 saturation of ammonium sulfate, since it was found that the product of the digestion of pTyrGel by collagenase does not precipitate at this ammonium sulfate concentration. This method is also adequate for the removal of the collagenase from the antibodies, since the enzyme is still

[1] Abbreviations used in this work: pTyrGel, poly-L-tyrosyl gelatin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; CM, carboxymethyl-; DEAE, diethylaminoethyl-.

soluble at this concentration of ammonium sulfate (Seifter *et al.*, 1959).

Digestion of ^{14}C-labeled pTyrGel by collagenase (1 unit/1 mg pTyrGel) was carried out in 0.05 M Tris buffer, *p*H 7.4, containing 0.005 M $CaCl_2$, for 3 hours at 37°.

Fragmentation of Antibodies by Water-insoluble Papain.—Water-insoluble papain was prepared as described previously (Cebra *et al.*, 1961) by coupling papain (twice crystallized, Worthington) with the product of the diazotization of a copolymer (Bar-Eli and Katchalski, 1963) of L-leucine and *p*-amino-DL-phenylalanine (residue molar ratio of 2.5:1). Hydrolysis of the antibodies by water insoluble papain was carried out for 10–20 minutes at *p*H 8.2 in 0.15 M NaCl and 0.002 M Versene (EDTA), at a protein concentration of 10 mg/ml. Insoluble enzyme was added to give the equivalent of 100 μg/ml of papain activity (Cebra *et al.*, 1961). After removal of the insoluble enzyme by filtration, thioglycolate (thioglycolic acid solution brought to *p*H 8.2 with sodium hydroxide) was added to the papain-treated antibodies up to a concentration of 0.01 M. The solution was incubated for 3 hours at 37°, and after a further 12 hours at 0° most of fragment III crystallized out. The crystals were centrifuged off, washed with 0.1 M sodium phosphate buffer, *p*H 7.0, dissolved in 0.05 M acetic acid, and after dialysis against 0.02 M acetic acid they were lyophilized. The supernatant was dialyzed against 0.01 M acetate buffer of *p*H 5.5 and chromatographed on a carboxymethylcellulose (CM) (Bio-Rad, 0.7 meq/g) column (Porter, 1959) to separate fragments I and II and the remnant of fragment III. The fractions obtained from the column were dialyzed against 0.02 M acetic acid and lyophilized. The reactions were followed routinely by sedimentation in the ultracentrifuge of both the intact rabbit antibodies and their fragments.

Fragmentation of Antibodies by Reduction.—The antibodies obtained by the digestion of pTyrGel–anti-pTyrGel precipitates with collagenase were separated from the free degradation products of the antigen on a Sephadex G-100 column at neutral *p*H. The antibody fraction was lyophilized and reconstituted in 0.5 M Tris buffer, *p*H 8.2. Reduction was carried out in 0.75 M mercaptoethanol (Organic Research Chemicals, Poyle Estate, Engl.), according to Fleischman *et al.* (1962). The sulfhydryl groups were then alkylated by adding iodoacetic acid (Eastman Kodak, recrystallized from ether–petroleum ether before use) as follows: the iodoacetic acid in aqueous solution was brought to *p*H 8.2 with sodium hydroxide and added to the protein solution at 0°. The whole reaction mixture was maintained at *p*H 8.2 by the addition of 1 N NaOH. The carboxymethylated product was dialyzed against 0.14 M NaCl for 4 hours and against 1 N propionic acid for 12 hours. It was then passed through a column of Sephadex G-100 or G-200 equilibrated with 1 N propionic acid at 4°. The fractions obtained from the Sephadex G-100 column were dialyzed against 0.14 M NaCl for 4 hours, concentrated by ultrafiltration (Hofsten and Falkbring, 1960), and tested by agar-gel diffusion with sheep antiserum against rabbit γ-globulin (Burroughs Wellcome, Engl.). Only fraction A gave a line with this antiserum.

Antisera.—Rabbits were immunized against pTyrGel by injecting the antigen in complete Freund's adjuvant (Difco) according to the procedure described by Sela *et al.* (1962). Quantitative complement-fixation measurements and quantitative precipitin tests were performed according to Kabat (1961). The washed precipitates were dissolved in 0.1 N NaOH and the amount of antigen present was determined by radioactivity (after trace-tagging the antigen either with radioactive iodine or by using antigen prepared with ^{14}C-tyrosine), or by determination of hydroxyproline after hydrolysis. The amount of antibody in the precipitates was obtained from the optical density at 280 mμ after making the necessary corrections for the contribution of the antigen to the absorbancy.

γ-Globulin and Antibodies.—Rabbit γ-globulin was prepared by precipitation from pooled rabbit serum at 0.4 saturation of ammonium sulfate at 5° and subsequent chromatography on a DEAE-cellulose (Eastman Kodak) column, under the conditions described by Levy and Sober (1960).

Antibodies to lysozyme were purified immunospecifically according to Givol *et al.* (1962). Antibodies to ovalbumin were a gift from Dr. A. T. Jagendorf, who purified them making use of a specific immunoadsorbent prepared by coupling ovalbumin with the diazotization product of a copolymer of L-leucine and *p*-amino-DL-phenylalanine.

Immunoadsorbent.—Water-insoluble immunoadsorbent for antibodies with specificity toward tyrosine peptides was prepared by coupling (Sela and Katchalski, 1955) poly-L-tyrosine of a degree of polymerization, $n = 9$, with the diazotization product of a copolymer (Bar-Eli and Katchalski, 1963) of L-leucine and *p*-amino-DL-phenylalanine.

Immunodiffusion.—Gel-diffusion tests were carried out at room temperature in 1.5% agar (Difco) and 0.5% NaCl by the technique of double diffusion on agar plates (Ouchterlony, 1953). Sheep antiserum against rabbit serum or against rabbit γ-globulin (Burroughs Wellcome, Engl.) was used to identify the rabbit antibodies.

Electrophoresis.—Paper electrophoresis was performed on Whatman No. 1 paper in a Veronal buffer of *p*H 8.6, and ionic strength 0.1. Samples were processed for 16 hours at room temperature in a voltage gradient of 3 v/cm.

Ultracentrifugation.—Sedimentation analyses were performed in a Spinco Model E ultracentrifuge at 56,100 rpm. Diffusion measurements were made in the same centrifuge with a synthetic-boundary cell according to Ehrenberg (1957).

Determination of Protein Concentration.—Protein concentration was determined by optical density measurements at 280 mμ in a 1-cm cell in a Beckman DU spectrophotometer.

Determination of Hydroxyproline.—Hydroxyproline was determined in the hydrolysates of the antigen-antibody precipitates as well as in the hydrolysates of the antibodies by the method of Neuman and Logan (1950).

Measurements of Radioactivity.—Radioactivity of ^{131}I-labeled antigen or its degradation products was measured in a well-type Tracelab scintillation counter. Radioactivity of ^{14}C-labeled antigen was measured in a thin-window gas-flow counter (Nuclear Chicago).

Results

Isolation and Characterization of the Antibodies.—The immunological systems of three different polytyrosyl gelatin derivatives were investigated by Arnon and Sela (1960a). While attachment of small amounts of tyrosine to gelatin enhanced its antigenicity considerably, no significant increase was found in antibody production in rabbits when the tyrosine content of the antigen was increased from 5.7% to 10.3%. In agreement with this result we found

FIG. 1.—Paper electrophoresis of antibodies to pTyrGel. The antibodies used were obtained by digestion of the immunological precipitate with collagenase and removal of the free degradation products of the antigen (see text). Electrophoresis of the antibodies and normal serum was performed in Veronal buffer of *p*H 8.6 and ionic strength 0.1, at 3 v/cm for 16 hours. The line of application is on the left side. The strips were stained with amido black 10 B.

that pTyrGel containing 16% tyrosine residues (5.1 tyrosine residues, on the average, per peptide chain attached to gelatin; this number was calculated assuming 23 moles of distinct polypeptide chains per 100,000 g of gelatin [Sela and Arnon, 1960]) does not elicit significantly more antibodies in rabbits than was elicited by pTyrGel containing 10.3% tyrosine residues. Different antisera used in this study contained 0.4–0.6 mg precipitable antibodies per 1 ml serum.

A typical feature of the pTyrGel–anti-pTyrGel systems with 5.7% or 10.3% tyrosine residues in the antigen was that under conditions of maximal precipitation of antibodies only 20–30% of the added antigen was present in the precipitate, while most of the remaining antigen was found in the supernatant in the form of soluble complexes with antibodies (Arnon and Sela, 1960a). Also in the case of pTyrGel containing 16% tyrosine residues only 30% of the antigen was present in the precipitate under conditions of maximal antibody precipitation.

By quantitative complement-fixation analysis it was found that upon reaction of pTyrGel (16% Tyr) with 1 ml of its antiserum, 80 hemolytic units ($C'H_{50}$) were fixed from guinea pig serum in the region of maximum antibody precipitation.

Arnon and Sela (1960a) have shown by inhibition studies that in pTyrGel containing 10.3% tyrosine residues the antigenic specificity resides in the tyrosine peptide chains (3.2 tyrosine residues on the average) attached to the amino groups of gelatin. When an antiserum to pTyrGel (16% Tyr) was treated for 1 hour at 37° with an insoluble material prepared by coupling polytyrosine with the diazotization product of a copolymer of L-leucine and *p*-amino-DL-phenylalanine, the antibodies were completely removed from the serum. This finding provides further support for the suggestion that the antigenic determinant sites of pTyrGel (16% Tyr) are the tyrosine peptides attached to the gelatin molecule. The antibody activity of the antiserum to pTyrGel (16% Tyr) was associated with the globulin fraction obtained by precipitation of the antiserum at 0.4 saturation of ammonium sulfate, or with the γ-globulin fraction of the antiserum fractionated on a DEAE-cellulose column (Levy and Sober, 1960).

In order to further characterize the antibodies, the method of selective proteolysis of pTyrGel–anti-pTyrGel precipitates (Arnon and Sela, 1960b) was chosen for their isolation. The immunological precipitate formed by mixing 74 ml of antiserum and 14.8 mg of ^{14}C-labeled pTyrGel was washed with

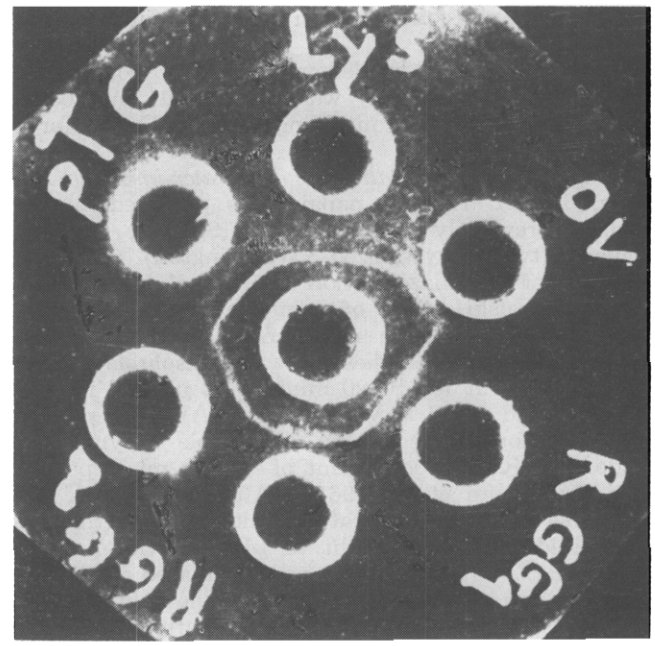

FIG. 2.—Agar-gel diffusion of purified antibodies. The central well contains antiserum from sheep against rabbit serum. Lys = antibodies to lysozyme purified as described by Givol *et al.* (1962); pTG = antibodies to pTyrGel isolated as described in Fig. 1; OV = antibodies to ovalbumin purified by use of a specific immunoadsorbent (see Experimental); RGG_{-1}, RGG_2 = two different preparations of rabbit γ-globulin.

0.14 M NaCl and treated in Tris buffer (4 ml) with collagenase (5 units), as described under Experimental. After 1 hour most of the precipitate was dissolved and after 2 more hours the small precipitate that still remained was centrifuged off and discarded. The supernatant was dialyzed exhaustively against 0.14 M NaCl to remove the free degradation products of the antigen.

The antibodies inside the dialysis bag still contained 58% of the radioactivity that was present in the original precipitate. By sedimentation analysis they moved as a single peak of 6.5 S. On paper electrophoresis they migrated identically with the γ-globulin of rabbit normal serum (Fig. 1). Upon immunodiffusion (Fig. 2) against sheep antiserum to rabbit serum, the antibodies to pTyrGel gave a common line with rabbit γ-globulin as well as with immunospecifically purified antibodies to lysozyme and ovalbumin.

The addition of new pTyrGel to the above antibody preparation did not result in any precipitation. In order to test the possibility that the antibody-combining sites are blocked by the antigenic determinant pieces that are still bound to them (Arnon and Sela, 1960b), an attempt was made to remove these pieces of antigen from the antibodies by passing the preparation through a Sephadex G-50 column under conditions known to dissociate antigen-antibody bonds (acid *p*H). The results of this experiment as well as of passage through similar columns, at a neutral *p*H value, of the same antibody preparation or of normal rabbit γ-globulin mixed with radioactive fragments obtained by the digestion of pTyrGel with collagenase, are given in Figure 3. The degradation products of ^{14}C-labeled pTyrGel could indeed be separated completely from rabbit γ-globulin on the Sephadex column (upper curve). However, when a solution derived from an immunological precipitate of pTyrGel by digestion with collagenase was passed through a Sephadex G-50 column at neutral *p*H, 47% of the labeled pieces of

the antigen remained bound to the antibodies (middle curve). On passing a similar solution through a Sephadex column at *p*H 3 (bottom curve) only 14.7% of the radioactivity was associated with the antibody fraction, indicating dissociation of 69% of the labeled antigenic pieces.

The method described above for the preparation of antibodies to pTyrGel by means of the digestion of the immunological precipitates with collagenase, was repeated on a larger scale, using iodination with ^{131}I for labeling the antigen.

Antibodies from 300 ml of antiserum were precipitated by the addition of 30 mg of the labeled antigen. The washed precipitate was digested with collagenase. From the radioactivity and optical density of the solution obtained it was calculated that the precipitate contained 13.2 mg of antigen and 110 mg of antibodies. The antibodies were precipitated from this solution at 0.37 saturation of ammonium sulfate at 0°. Under these conditions neither collagenase nor the free degradation products of pTyrGel precipitated. The precipitate was centrifuged, washed with a cold 0.37 saturated ammonium sulfate solution, dissolved in 0.14 M NaCl, and dialyzed extensively against this solvent. It was found to contain 63% of the radioactivity and 15% of the hydroxyproline that were present in the original precipitate. Passage of this solution through a Sephadex G-100 column (43 × 2.5 cm) at *p*H 1.8 (0.14 M NaCl–0.02 M HCl) resulted in an almost complete separation of the antibodies from the radioactive antigenic pieces (90% dissociation). The antibody fraction thus obtained was brought to *p*H 7.0, dialyzed against 0.14 M NaCl, and analyzed by sedimentation (s_{20w} = 6.5 S) and diffusion (D_{20} = 3.75 × 10^{-7} cm^2 sec^{-1}) in the ultracentrifuge. The molecular weight of the antibodies, calculated from these data, is 160,000.

The purified antibodies were found to give a typical precipitin curve upon addition of fresh antigen. In the region of maximal antibody precipitation, 60% of the antibody was found in the precipitate.

From the weight ratio of antigen and antibody in the immunological precipitate in the equivalence zone, the tyrosine content of the antigen (16% tyrosine residues), the molecular weight of the tyrosine residue (163), the molecular weight of the antibody (160,000), and the amount of radioactivity (labeled tyrosine) that remained bound to the antibodies after removal of the free degradation products (63%), a value of 5.9 moles of tyrosine residues per mole of antibody-combining site was calculated, assuming two combining sites per antibody molecule. This is in good agreement with the calculated average number, 5.1, of tyrosine residues in each of the peptides attached to the gelatin in pTyrGel containing 16% tyrosine residues, suggesting that an antigen segment containing one such tyrosine peptide is bound to each combining site in the antibody.

In the following section experiments are described which are concerned with location of the area of the antibody to which the antigen piece is bound.

Fragmentation of the Antibodies by Papain.—The basic findings of Porter (1959) showed that rabbit γ-globulin and antibodies can be split by cysteine-activated papain into three 3.5 S fragments which are separable on a CM-cellulose column. Two of these fragments (fragments I and II) bear the antibody activity and are univalent, while the third (the crystallizable fragment III) is devoid of antibody activity. Cebra *et al.* (1961), using a water-insoluble papain derivative, showed that the fragmentation of rabbit γ-globulin by papain could be resolved into

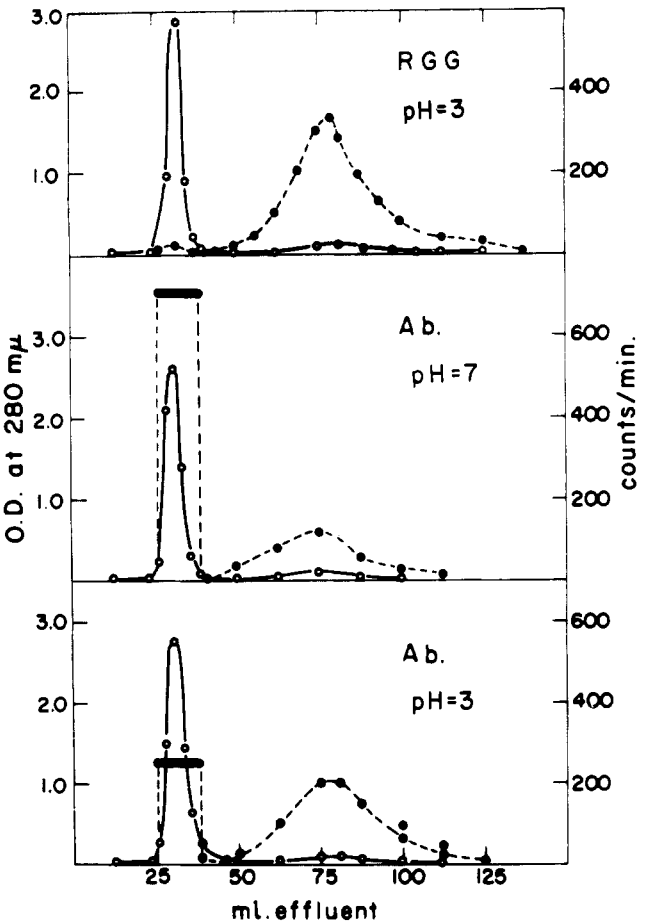

FIG. 3.—Purification of antibodies to pTyrGel on a Sephadex G-50 column. *Top:* Rabbit γ-globulin (10 mg) mixed with a digest of ^{14}C-labeled pTyrGel (1.1 mg) by collagenase. Elution from the column with 0.14 M NaCl adjusted to *p*H 3.0 with 1 N hydrochloric acid. *Middle:* Antibodies to pTyrGel (10 mg) isolated by splitting the immunological precipitate with collagenase and still containing ^{14}C-labeled pieces of the antigen (1.1 mg). Elution from the column with 0.14 M NaCl adjusted to *p*H 7.0 with 0.1 N sodium hydroxide. *Bottom:* As in the middle curve, except that elution from the column was performed with 0.14 M NaCl at *p*H 3.0. The Sephadex column (70 × 1.5 cm) was equilibrated before each experiment with the eluting solvent. In the experiments shown in the middle and bottom parts of this figure the radioactivity in the antibody fractions was counted only for the pooled tubes. ○ = optical density; ● = radioactivity.

two stages, first limited proteolysis and then reduction. Thus, hydrolysis of 3–5 peptide bonds suffices to render the 6.5 S γ-globulin susceptible to fragmentation by reduction into the above 3.5 S fragments. We used this method for the fragmentation of the antibodies to pTyrGel isolated by the procedure making use of collagenase, and still bearing ^{131}I-labeled pieces of the antigen. The fragments of the antibody molecule binding these pieces were followed by means of their radioactivity. A quantity of 100 mg of antibodies carrying ^{131}I-labeled pieces of pTyrGel was isolated as described in the last experiment of the previous section. These antibodies were treated with water-insoluble papain as described under Experimental, and after removal of the insoluble enzyme, they were reduced in 0.01 N thioglycolate, *p*H 8.0, at 37° for 2 hours. After a further 12 hours at 0° the crystals of fragment III (19 mg containing only 3.8% of the radioactivity) were centrifuged off and washed. When these crystals were dissolved in 0.02

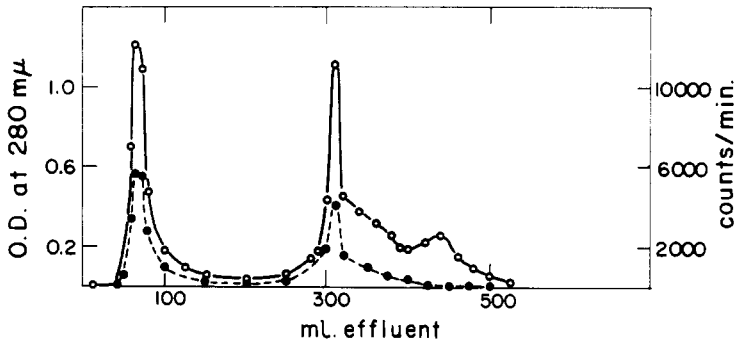

FIG. 4.—Chromatographic separation of fragments from antibodies to pTyrGel on a CM-cellulose column. The antibodies, containing bound pieces of ^{131}I-labeled pTyrGel (see text), were digested by water-insoluble papain and subsequently reduced. Chromatography of the fragments (3.5 S) was performed after removal of the crystals of fragment III. Dimension of the column, 25 × 2.5 cm; conditions of chromatography as described by Porter (1959); the point of application of the gradient is indicated by the arrow. ○ = optical density; ● = radioactivity (cpm).

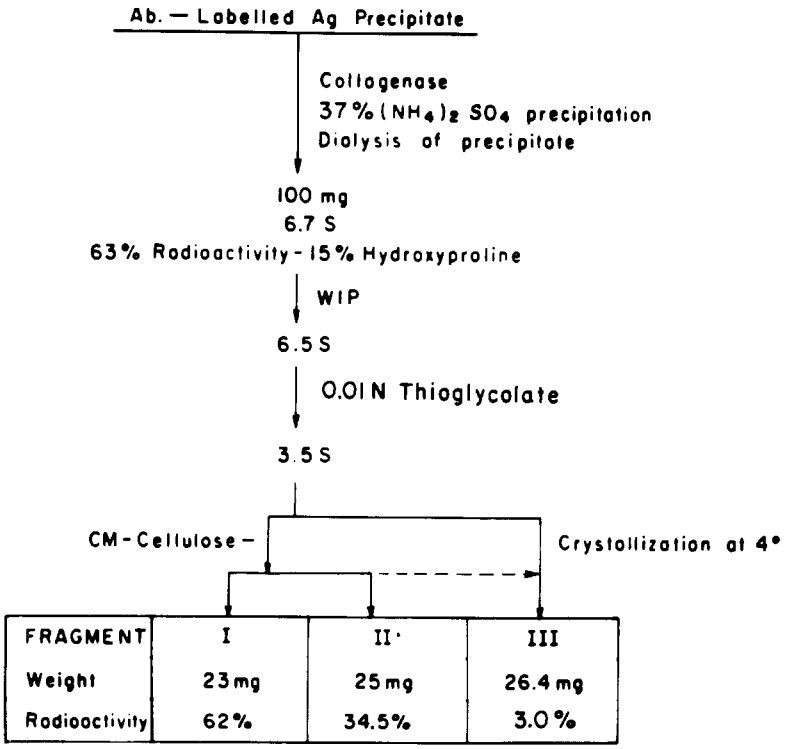

FIG. 5.—A flow chart describing the experiment on the fragmentation of antibodies to pTyrGel by water-insoluble papain (WIP) followed by reduction.

M acetic acid and analyzed in the ultracentrifuge, only one peak of 3.2 S was observed.

The supernatant (81 mg) gave one peak of 3.7 S upon sedimentation in the presence of the reducing agent; it was dialyzed against 0.01 M acetate buffer, *p*H 5.5, and applied to a CM-cellulose column equilibrated with the same buffer (Porter, 1959) for the separation of fragments I and II and the remnants of fragment III. Figure 4 shows the elution pattern from this column as followed by measurements of optical density and radioactivity. Figure 5 is a flow chart showing the details of this experiment. It is shown that 97.0% of the radioactivity (i.e., labeled antigen pieces) is associated with fragments I and II, known to contain the antibody-combining site. Thus, it may be concluded that the antigenic pieces which are bound to the antibodies and block their activity (see the previous section) are associated with the antibody-combining sites of fragments I and II, and that fragment III, which does not bind radioactive antigen pieces, is devoid of antibody-combining sites. This conclusion is in agreement with the original findings of Porter (1959).

Reduction of the Antibodies.—Fleischman *et al.* (1962) recently reported the successful separation on Sephadex G-75 of two fractions (A and B) from rabbit and other γ-globulins reduced with mercaptoethanol in the absence of denaturing agents. In the case of rabbit immune γ-globulin they showed that fraction B, comprising approximately 25% of the γ-globulin, does not display any antibody activity although it has an antigenic component in common with fragment I (obtained by papain hydrolysis of the γ-globulin). Fraction A, on the other hand, has antigenic determinants in common with both fragment I and fragment III, and exhibits antibody activity. However, the recovery of antibody activity was relatively low and it could be demonstrated only by coprecipitation with the unreduced antibody and antigen (Porter, 1962; Fleischman *et al.*, 1963).

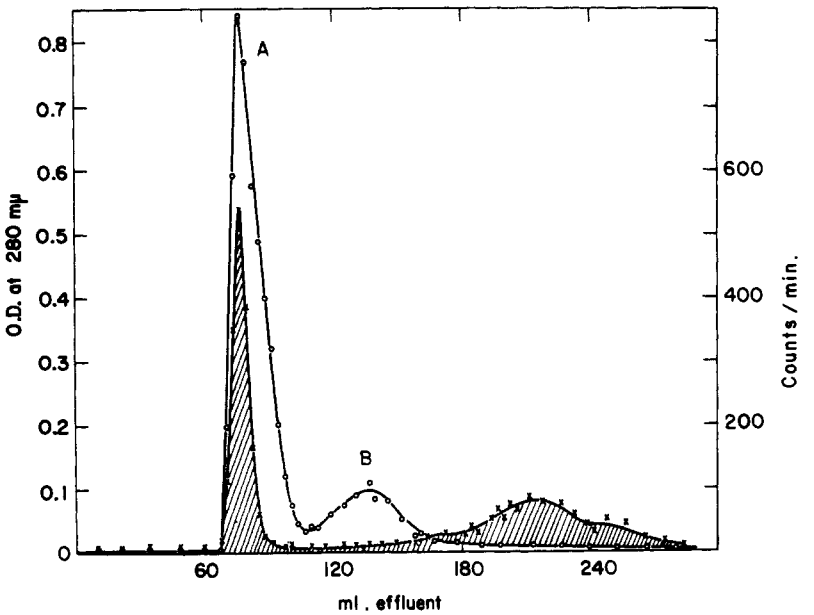

FIG. 6.—Separation of fractions A and B from antibodies to pTyrGel. The antibodies containing bound ^{14}C-labeled pieces of pTyrGel were reduced, carboxymethylated, and dialyzed against 1 N propionic acid. The product (16.5 mg) was applied to a Sephadex G-100 column (45 × 3 cm i.d.). Elution from the column was made in 1 N propionic acid. ○ = optical density; × = radioactivity.

We have attempted to obtain information as to the antibody activity of fractions A and B by reduction under the conditions of Fleischman *et al.* (1962), of antibodies labeled with radioactive pTyrGel pieces. The antibodies were prepared by the digestion of a ^{14}C-labeled pTyrGel-anti-pTyrGel precipitate with collagenase, followed by removal of the free degradation products by passing the solution thus obtained through a Sephadex G-100 column in 0.05 M sodium phosphate buffer, *p*H 7.0, at 4°. The separation of fractions A and B of reduced γ-globulin is performed in 1 N propionic acid, conditions under which considerable dissociation of antigen-antibody complexes occurs; therefore, a preliminary control experiment was performed in which the antibodies isolated as described above were dialyzed against 1 N propionic acid and passed through a Sephadex G-100 column equilibrated with 1 N propionic acid. The antibodies emerging from the column still contained 17% of the radioactivity that was originally present in the isolated antibodies.

In a separate experiment, the isolated antibodies were reduced and carboxymethylated as described under Experimental. After dialysis against 0.9% NaCl (4 hours) and 1 N propionic acid (12 hours) the material obtained (8.3 ml containing 16.5 mg) was applied to a Sephadex G-100 column that was equilibrated with 1 N propionic acid at 4°. The chromatographic procedure, illustrated in Figure 6, shows the separation of fractions A and B. Fraction B (comprising 25% of the material) was found to be almost free of radioactivity. The ^{14}C-labeled pieces of the antigen emerged from the column either together with the protein of fraction A or as free peptides appearing after the protein of fraction B (probably pieces of the antigen which were dissociated under the conditions of fractionation). The protein in fraction A contained 13% of the radioactivity initially bound to the intact antibodies. However, it is apparent from Figure 6 that the radioactivity is associated with the first part of the peak containing fraction A. This result implies that the fraction A obtained in this experiment is not homogeneous. In order to find conditions for better separation of the proteins in fraction A, the reduced antibodies (18 mg) were applied to a Sephadex G-200 column (50 × 3 cm) under the above conditions. While the small amount of material taken did not permit a detailed analysis of the fractions obtained, it was found that the protein emerging as fraction A in the previous experiment was resolved into two fractions and that the radioactivity was associated only with the first of these fractions.

DISCUSSION

Antibodies to poly-L-tyrosyl gelatin preparations possessing around 15% tyrosine residues may be isolated by making use of digestion with collagenase of the pTyrGel in the immune precipitates. The antibodies purified in this way still contain pieces of the antigen bound to their combining sites. Similar yields of the bound antigen pieces, after extensive dialysis of the antibodies, were obtained in experiments in which the antigen was tagged with ^{14}C in the tyrosine residues (58%) or trace-labeled with radioactive iodine (63%). The agreement is not surprising as, in view of the low content of histidine in gelatin (0.7%; Eastoe, 1955), the iodine must have reacted almost exclusively with the tyrosine residues of the polytyrosyl gelatin.

In the intact antigen molecule the tyrosine and hydroxyproline contents were 16% and 11.2%, respectively. The radioactivity data and hydroxyproline analysis of the purified antibody preparations, in which pieces of polytyrosyl gelatin are still bound to the antibody, show that these antigen pieces still contain 63% of the tyrosine and 15% of the hydroxyproline of the intact antigen. Thus the antigen pieces attached to the antibody contain relatively much more tyrosine and much less hydroxyproline than the intact antigen (the ratio of Tyr:Hypro in the intact antigen is 1.4 while in the antigen pieces bound to antibody it is 4.2). This suggests that, as might be expected, the antigen molecule associates with the antibody molecule predominantly through its tyrosine peptide chains, and, consequently, that the antibody-combining sites have their specificity directed towards such chains. This conclusion is corroborated by the experiments

in which antibodies to polytyrosyl gelatin were removed quantitatively from the antiserum by polytyrosine cross-linked with the diazotization product of a copolymer of leucine and *p*-aminophenylalanine.

Additional information as to the nature of the antigenic determinants of polytyrosyl gelatin was obtained from an evaluation of the number of tyrosine residues bound, on the average, to each antibody site. The number obtained, 5.9, is in good agreement with the average chain length, 5.1, of tyrosine peptides attached to gelatin, and thus strongly suggests that each antibody-combining site associates with one tyrosine peptide chain of the antigen. Further experiments are needed in order to establish the size of the tyrosine peptide which is in direct contact with the reactive area of the antibody.

The pieces of antigen bound to the antibodies could be released only at acid *p*H values (Fig. 3), conditions known to dissociate antigen-antibody bonds. While the antibodies containing the pieces of antigen did not precipitate on addition of fresh antigen, a precipitin reaction could be demonstrated with the purified antibodies after they had passed through a Sephadex column at an acid *p*H. These isolated antibodies had the antigenic and physical properties of rabbit γ-globulin, and a molecular weight of 160,000.

Before removal of the labeled antigenic pieces it was possible to use them to follow the fragments of antibody which combine with them. Thus, after fragmentation of the purified labeled antibodies with water-insoluble papain followed by reducing agent, fractionation revealed that only fragments I and II contained pieces of the antigen. This is in agreement with several other reports that only these fragments carry antibody activity (Porter and Press, 1962), and gives further support to the suggestion that the pieces of antigen are indeed bound to the antibody-combining site. The observation that the labeled pieces of antigen are not distributed equally between fragments I and II raises some questions concerning the affinity of each of these fragments for the antigen, but no explanation is as yet available for this observation.

In view of the finding that the pieces of antigen are associated with active fragments of the antibody after fragmentation by papain, an attempt was also made to trace the antibody-combining sites by this technique after reductive cleavage of disulfide bridges in antibodies to polytyrosyl gelatin. The results described by Porter (1962) and by Fleischman *et al.* (1962) lead to the conclusion that among the fractions (A and B) obtained from rabbit or horse immune γ-globulin by reduction in the absence of denaturing agents, only fraction A carries antibody activity. Reduction in the presence of a denaturing agent (8 N urea) dissociated the γ-globulin molecule into two classes of polypeptide chains which can be separated on starch-gel electrophoresis in 8 M urea into light (L) and heavy (H) chains (Edelman and Poulik, 1961). Edelman *et al.* (1961) showed that when different purified guinea pig antibodies were compared by this method, differences in the starch-gel electrophoretic patterns are observed only among the light chains. Even though antibody activity could not be tested because of the insolubility of the reduced products in the absence of urea, it was suggested that the differences in the electrophoretic mobility of the L chains are correlated with differences in the specificity of the various antibodies. When fractions A and B of horse and human γ-globulin were examined by starch-gel electrophoresis under the same conditions as were used by Edelman *et al.* (1961), it was found that fraction B (which is devoid of antibody activity) migrates similarly to the L chains (Porter, 1962). Franek and Nezlin (1963) reported recently that by mixing together the A and B fractions they could restore the antibody activity which neither of them possessed separately, and concluded that for reaction with antigen a complex of A and B is required.

From experiments reported in this paper it may be inferred that fraction B, obtained upon reduction of antibodies in the absence of denaturing agents (Fig. 6), is completely devoid of antibody activity. On the other hand, radioactive antigen pieces were found to be associated with the front part of fraction A. This may imply that the protein in fraction A indeed possesses antibody activity. However, two other possibilities may be considered: (1) Two A chains devoid of antibody activity may combine to form a dimer (Fleischman *et al.*, 1962) which may possibly be capable of binding antigen. (2) It is also possible that while most of the bound pieces of the antigen were released from the antibodies in 1 M propionic acid, the relatively few remaining antigen pieces were instrumental in keeping A fragments associated with B fragments in the form of a complex with active antibody sites. This second alternative presupposes that the behavior in 1 M propionic acid of reduced antibody molecules bound to pieces of antigen is completely different from that of similar antibody molecules which are devoid of such antigen pieces. The possibility that these antigen pieces may have prevented the reductive cleavage of the antibody molecules may be eliminated, as the yields of A and B fractions from antipolytyrosyl gelatin obtained by reduction in the presence of antigen pieces was similar to the yields reported for rabbit γ-globulin unassociated with other molecules (Fleischman *et al.*, 1962).

The use of tagged antigen pieces to label the active sites of the antibodies may thus be of interest in studies on the activity of such sites following enzymatic or chemical fragmentation of antibody molecules and may help in their location.

Acknowledgment

The technical assistance of Mr. Moshe Rotman is gratefully acknowledged.

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A Comparison of Fragments of Rabbit Antibodies and Normal γ-Globulin by the Peptide-Map Technique*

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Received October 21, 1963

Rabbit γ-globulin and immunospecifically purified antibodies to lysozyme and to poly-L-tyrosyl gelatin were subjected to fragmentation by hydrolysis with water-insoluble papain followed by reduction. Fragments I and II and the crystalline portion of fragment III were isolated, reduced in 8 M urea, and carboxymethylated. These preparations were digested with pepsin or nagarse and the digests were used for a comparison of the peptide maps obtained upon two-dimensional chromatography and electrophoresis. The peptide maps of fragments I and II are very similar and differ from that of fragment III. Sugar-containing peptides were detected only in the peptide map of fragment III. The peptide maps of fragment III from γ-globulin and antibody are practically identical. The peptide maps of the active antibody fragments (fragment I or II) from γ-globulin and the above two antibodies are also very similar, but differences in a few peptides could be detected.

Many theories have been proposed to account for the formation of specific antibodies. All of them have to take into consideration the great chemical and physicochemical similarities between different immunospecific antibodies as well as between them and normal γ-globulin within the same species. At the same time they should be able to explain the great power of biological discernment that the antibodies exhibit in their reactions with antigens. From the chemical point of view one might distinguish between theories based on the assumption that the covalent structures of different antibodies are identical and their specificity is due to conformational change within the γ-globulin molecule (e.g., Pauling, 1940; Haurowitz, 1952), and those that assume that differences in the biological specificities of antibodies may be correlated with differences in their primary structure (e.g., Lederberg, 1959). Such differences would quite possibly be limited to the combining areas of the antibodies, which are only a small fraction of the antibody molecule (Kabat, 1961). It is therefore not surprising that the comparison of several immunospecifically purified antibodies revealed no significant differences in their amino acid composition (Smith *et al.*, 1955; Fleischer *et al.*, 1961). On the other hand, Koshland and Englberger (1963) have recently reported small but significant differences in the amino acid composition of antibodies to two different haptenic groups.

The technique of peptide mapping, by which peptides obtained by enzymatic digestion of a protein or its derivative are separated on paper in two dimensions by means of chromatography and high-voltage electrophoresis, has been remarkably successful in picking out very small differences of amino acid sequence between normal and pathological hemoglobins (Ingram, 1956, 1961), and has been since used extensively in studies on proteins of relatively low molecular weight. The technical difficulties increase rapidly with the size of the protein.

Gitlin and Merler (1961a) have compared the peptide patterns of rabbit antibodies specific for different types of pneumococcal polysaccharide using as starting material the specific precipitate or the antibody dissociated from it by strong salt solution. Antibodies, oxidized with performic acid, heat denatured or in the native form, were digested with subtilisin, trypsin, or chymotrypsin, and subjected to electrophoresis and chromatography. Depending on the pretreatment of the antibodies, either no differences were observed between the peptide maps obtained from different antibodies or differences were found which were small but reproducible. Fragments obtained by papain digestion of the antibodies (Porter, 1959) were similarly treated and compared. In this case some differences were found between the peptide patterns of digests of performic acid–oxidized fragments I obtained from different antibodies, and similar comparison of performic acid–oxidized fragments II also revealed differences. Gurvich *et al.* (1961) compared the peptide maps obtained by digestion with trypsin followed by chymotrypsin of native or denatured rabbit γ-globulin and rabbit antibodies to horse serum albumin. A difference of one peptide spot was observed.

In the present paper we report the results of a comparison of rabbit γ-globulin, antibodies to a native protein, lysozyme, and antibodies to an artificial

* This investigation was supported in part by a U. S. Public Health Service research grant (AI-04715) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Presented in part at the 143rd Meeting of the American Chemical Society in Cincinnati, Ohio (Sela *et al.*, 1963a).