IMMUNOLOGICAL STUDIES OF HUMAN $\gamma$-GLOBULIN

RELATION OF THE PRECIPITIN LINES OF WHOLE $\gamma$-GLOBULIN TO THOSE OF THE FRAGMENTS PRODUCED BY PAPAIN

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(Received for publication, March 9, 1960)

Although human $\gamma$-globulin has been shown to be heterogeneous by a variety of physical, chemical, and biological criteria, immunologic analyses using anti-$\gamma$-globulin sera in gel diffusion techniques usually reveal only a single precipitation line. In certain instances additional lines are seen which are clearly due to the minor constituents of $\gamma$-globulin or to contaminating proteins. In other cases double lines have been observed which are not readily explicable on this basis and several hypotheses have been proposed to explain their origin.

Williams and Grabar (1), in an immunoelectrophoretic study of normal $\gamma$-globulin, have described the appearance of double $\gamma$-globulin precipitation lines under conditions of antibody excess. These authors did not conclude that doubling of the precipitation line resulted from antigenic heterogeneity, but surmised that the technical conditions of their experiments were responsible.

Recent observations by one of the present authors (2) have revealed double $\gamma$-globulin lines in immunoelectrophoretic analyses of a variety of normal and pathological sera. The present investigation was undertaken when several antisera were encountered which gave particularly clear cut double bands by Ouchterlony plate and immunoelectrophoretic procedures when tested against a wide variety of highly purified 7S $\gamma$-globulin preparations. These antisera were made against pathological $\gamma$-globulins, but gave double lines with normal $\gamma$-globulins. The possibility arose that these lines might reflect different antigenic determinants on $\gamma$-globulin molecules.

The development by Porter (3) of a method to split $\gamma$-globulin using papain has made it possible to explore the antigenic constitution of $\gamma$-globulin in greater detail. The use of this enzymatic method, in conjunction with immunoelectrophoretic analysis of the split products, has lead to the demonstration of separate antigenic determinants related to the multiplicity of precipitation lines obtained with whole $\gamma$-globulin.

Materials and Methods

1. Gamma Globulin.--A variety of $\gamma$-globulin samples (Fraction II) were used. One lot (Lederle C383) was purified further by chromatography on carboxymethylcellulose. In several
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instances normal human serum was also submitted to immuno-electrophoretic analysis. In one experiment, Fr. II \( \gamma \)-globulin was separated into euglobulin and pseudoglobulin fractions for comparative analysis. Gamma globulins of different electrophoretic mobilities were prepared by separation of Fr. II by zone electrophoresis as described previously (4). The slowly migrating and fast migrating portions of the \( \gamma \)-globulin peak were separated for use in some of the experiments.

2. Antisera.—Eighteen different antisera were used for this study. Three antisera from horses immunized with whole normal human serum were obtained from the Pasteur Institute in Paris (H-13, H-511, and H-491). Six antisera were obtained from rabbits immunized with normal human Fraction II \( \gamma \)-globulin (Rb-Go, Rb-133, Rb-135, Rb-137, Rb-139, Rb-141). Antisera Rb-382 and Rb-383 were from rabbits immunized against electrophoretically isolated \( \gamma \)-globulin. The remaining seven antisera were obtained from rabbits immunized with electrophoretically isolated pathological proteins. Five of them (Rb-Be, Rb-La, Rb-Tr, Rb-L and Rb-Ma) were directed against \( \beta_2 \)-myeloma proteins (5). One antiserum (Rb-Gd) was directed against a \( \gamma \)-myeloma protein. The remaining antiserum (Rb-Sam) was obtained from a rabbit immunized with a pathological macroglobulin.

3. Enzymatic Treatment of \( \gamma \)-Globulin.—Papain, twice recrystallized, (Mann Laboratories), was used to treat the \( \gamma \)-globulin samples under exactly the conditions described by Porter (3).

4. Chromatography.—The ion exchangers employed were carboxymethylcellulose and diethylaminoethylcellulose (6) obtained from BIO-RAD Laboratories, Richmond, California. Gradient elution from CM-cellulose was performed, using Porter’s conditions (3). When DEAE-cellulose was used, elution was obtained with pH 7.68 sodium phosphate buffer employing a gradient from 0.007 to 0.7 M. To check the chromatographic patterns and to purify the material in the peaks, pooled fractions were taken and rechromatographed in the same system. Chromatography was performed at room temperature; when periods longer than several hours were necessary, buffers were saturated with toluene.

5. Electrophoretic Purification.—Fr. II \( \gamma \)-globulin and papain-treated \( \gamma \)-globulin were submitted to zone electrophoresis on starch, using methods previously described (4).

6. Gel Diffusion Methods.—
(a) Immuno-electrophoresis: Scheidegger’s micromethod of immuno-electrophoresis, using 25 × 75 mm. glass slides, was employed (7). A 2 per cent agar gel, made in barbital-NaCl buffer, pH 8.6 and ionic strength 0.025, was used as a medium for electrophoretic separation under a potential gradient of 4V per cm. Antigen solutions in amounts of 1 to 2 microliters were placed in wells at the origin. After electrophoretic separation was accomplished, antisera were introduced into 1 mm wide channels cut according to the requirements of the experiments. In most cases, diffusion was allowed to proceed for 24 hours, and the preparations were washed for 3 days with saline and distilled water. They were then dried, and stained with amidoschwarz or with nigrosin.

(b) Ouchterlony plates: The techniques utilized were those described previously (8).

7. Ultracentrifugal Analysis.—This was done with a Spinco model E analytical ultracentrifuge. Plates were analyzed using methods described by Trautman (9).

RESULTS

A. Immunological Analysis of Whole \( \gamma \)-Globulin

In Fig. 1 are shown the patterns developed against whole Fr. II \( \gamma \)-globulin, using various antisera. The line closest to the antibody well has been designated as the outer line, and that nearest the antigen source, the inner line. Of the

1 CM, carboxymethyl; DEAE, diethylaminoethyl.
Fig. 1. Diagrams of immunoelectrophoretic patterns of chromatographically purified whole \( \gamma \)-globulin and papain-split \( \gamma \)-globulin, using various antisera. The dotted lines refer to the fainter bands. The anode is on the left in each diagram.

Three horse antisera illustrated, one showed a distinct double precipitation line, the second showed only one line, and the third demonstrated splitting at the end of the line. In the group of rabbits immunized with normal \( \gamma \)-globulin, five animals produced antisera giving a single \( \gamma \)-globulin precipitation line.
Fig. 2. Precipitin lines with 4 antisera of chromatographically purified Fr. II γ-globulin (reading from top to bottom, they represent antisera H.511, Rh-141, Rh-Sam, Rh-Tr). The two lower patterns show double lines. The outer line in the lowest pattern is faintly reproduced.

Two rabbit antisera showed double lines of unequal intensities, the inner line in both cases being the fainter and appearing primarily at the ends. The remaining rabbit antiserum of this group showed a complex pattern consisting of at least three precipitation lines.
The group of rabbits immunized against β₂₅-myeloma proteins produced antisera two of which showed a single line, two of which showed clear double lines, and one of which showed multiple lines. The anti-γ-myeloma serum yielded one strong single line, while the antiserum against a pathological macroglobulin produced two unambiguous and clearly separated double precipitation lines.

When double lines were produced by antisera against pathological proteins, the spatial separation of the two lines was greater than that of similar patterns obtained from anti-normal γ-globulin antisera. Fig. 2 illustrates both single and double line patterns produced by different antisera with the same preparation of γ-globulin. Fig. 3 shows the double line pattern in Ouchterlony plates. Antiserum Rb-Sam which showed the best separation of the two lines was used in this case.

Considerable efforts were directed toward investigating the possibility that a separate type of γ-globulin was responsible for the second major line. Immunoelectrophoretic experiments indicated that both lines were strikingly similar in their broad mobility distribution, and that both extended from the γ-globulin area to the α₂ region. This finding made it highly unlikely that a contaminating protein could be responsible: it would have to possess this distinctive broad mobility property of γ-globulin. Subfractionation of Fr. II was attempted, employing Zn precipitation as well as zone electrophoresis. All preparations gave the double band; both fast and slowly migrating γ-globulin demonstrated this effect. Euglobulin and pseudoglobulin, separated by dialysis, had this property. The pure 7S fraction of γ-globulin prepared by density gradient centrifugation also showed this effect. The genetic γ-globulin groups
of Grubb (10, 11) were also investigated. Both Gm-positive and Gm-negative γ-globulin gave the double bands. Several myeloma proteins with immunological properties similar to those of normal γ-globulin also showed double bands with these antisera.

B. Immunological Analysis of Papain-Treated γ-Globulin (Unfractionated)

1. Fraction II γ-Globulin — The second column in Fig. 1 illustrates the results obtained by immunoelectrophoresis using papain-treated Fr. II γ-globulin, and the series of antisera discussed in section A. Although it is clear that the products of papain treatment retained the capacity to precipitate with most of the antisera, no precipitation lines could be obtained using Rb-La and Rb-Ma antisera. These antisera reacted only faintly with whole Fr. II γ-globulin.

The majority of the antisera yielded complex patterns differing in detail, some showing as many as five distinct lines, others showing only one precipitation line. In most cases, a constant feature was the presence of two major components. Eight rabbit antisera produced patterns consisting of these two components only. For the purpose of later discussion we will call these S (slow), and F (fast). It should be noted, however, that in some cases (H-491, Rb-137, Rb-Be, Rb-Gd, and Rb-Sam) a minor component, electrophoretically faster than F and cross-reacting with it, could be observed. Furthermore, antisera H-491, Rb-Be, and Rb-Sam showed a minor outer line parallel to the S component. Antiserum Rb-Go, in addition to demonstrating the S and F components, yielded a faint outer line with extensive electrophoretic distribution.

Figs. 4 a and 4 b contain photographs comparing patterns of whole γ-globulin with papain-treated globulin. Four different antisera were used. Comparison of the mobilities indicated that the S component had an electrophoretic distribution extending further toward the cathode than whole γ-globulin. The F component of papain-treated γ-globulin possessed a β-mobility.

Both crude Fr. II preparations as well as Fr. II which had been isolated from the center of the major peak obtained with carboxymethylcellulose chromatography, were treated with papain and studied with the various antisera. Similar results were obtained, the S and F components appearing in each case. The patterns in Fig. 1 were all obtained with the chromatographically purified Fr. II material.

The immuno-electrophoretic and Ouchterlony plate experiments demonstrated that the F and S components penetrated through each other, indicating marked antigenic differences. In most experiments with various preparations of degraded γ-globulin these differences appeared to be complete without any cross-reaction. In some experiments there appeared to be weakening of the F component on passing through the S line; the reverse was never observed. Thus, with certain antisera, the possibility of some slight cross-reaction was not completely ruled out.
FIGS. 4 a and 4 b. Comparison of the immunoelectrophoretic patterns of whole and split γ-globulin. The lower patterns in each case represent the split γ-globulin. (a) Antiserum H-491 in middle slit; antiserum H-511 in lateral slits. (b) Antiserum Rb-Sam in middle slit; antiserum Rb-137 in lateral slits.

Fig. 4 c. Immunoelectrophoretic patterns of fast-migrating γ-globulin (upper) and slowly migrating γ-globulin (lower) after papain treatment; antiserum used was Rb-382.
2. Electrophoretically Isolated $\gamma$-Globulin.—Whole Fr. II $\gamma$-globulin was submitted to zone electrophoresis on starch and yielded a single broad peak. The leading portion and the trailing portion of this peak were isolated, yielding two $\gamma$-globulin components of different mobilities as demonstrated previously (4). Rerunning of these components indicated these mobility differences; after mixing the components they gave two separate peaks. After papain treatment, these $\gamma$-globulins yielded the immunoelectrophoretic patterns illustrated in Fig. 4c. The F components were similar for the two, but the S component of the “slow” $\gamma$-globulin had a markedly lower mobility. The point of crossing of the F and S fractions was also quite different. In the experiment illustrated in Fig. 4c, the F and S components of the slowly migrating $\gamma$-globulin are too greatly separated to see the crossing. In another experiment in which there was greater separation of slowly migrating and fast migrating $\gamma$-globulin, a somewhat different pattern was observed after papain treatment. In this case the deg-
radation products of the slowly migrating \( \gamma \)-globulin showed a third component of intermediate mobility (see Fig. 8). This fused with the F fraction and penetrated through the S. The fast migrating \( \gamma \)-globulin gave essentially the same picture as in the experiment illustrated in Fig. 4 c.

3. Myeloma Proteins.—Five myeloma proteins which gave a single sharp peak on electrophoresis were isolated by zone electrophoresis on starch and submitted to papain treatment under the same conditions as described above for \( \gamma \)-globulin. Two of these, with mobilities in the \( \gamma \)-globulin range, were closely related to ordinary \( \gamma \)-globulin immunologically, and showed patterns very similar to those described above for \( \gamma \)-globulin. F and S components were observed which crossed through each other. This finding is illustrated in Fig. 5 a. The upper pattern shows the myeloma globulin before papain treatment, and the lower pattern represents the same protein after papain treatment. The central slit contained antiserum Rb-Tr which gave a double line with the myeloma protein and also developed the F and S components. The outer slits contained antiserum Rb-137 which gave a single line with whole myeloma protein, and in this instance reacted only with the S component. Fig. 5 b illustrates the pattern of another myeloma protein and shows the S, F, and midcomponents after papain treatment. Absorption with S component derived from normal \( \gamma \)-globulin left only the F and midcomponents. The close relation of the midcomponent to the F component is also apparent in this experiment. Two other myeloma proteins gave a somewhat different picture clearly showing the F components but only faint bands corresponding to S component. In addition, a midfraction fusing with the F was consistently observed. The fifth myeloma which was a \( \beta \)sa type (5) gave a very different pattern. The results with the different myeloma proteins were very complex and will be described in detail elsewhere. In the above experiments, antisera against normal \( \gamma \)-globulin were employed. Experiments with antisera to the individual myeloma proteins were not carried out.

C. Analyses of Chromatographically Isolated Components of Papain-Treated \( \gamma \)-Globulin

In Fig. 6 are shown diagrams illustrating the chromatographic separation of papain-treated Fr. II \( \gamma \)-globulin.

In diagram A is represented the chromatogram obtained using 100 mg. of papain-treated \( \gamma \)-globulin, applied to a 15 \( \times \) 2 cm. column of DEAE-cellulose. Tubes 5 and 20 of the original chromatogram (A) were removed for immunoelectrophoretic analysis before pooling the remaining tubes for rechromatography. The indicated areas were rechromatographed separately on 10 \( \times \) 0.9 cm. DEAE-cellulose columns using the same buffer gradient system. The results are shown in B and C. The peaks rechromatographed reproducibly and showed only a small amount of contaminating material. It should be
pointed out that on rechromatographing the second peak shown in A, partial resolution into two peaks was observed. Chromatography of papain-treated human \( \gamma \)-globulin on carboxymethylcellulose, using Porter’s conditions (3), did not resolve separate components.

![Diagram](image)

**FIG. 6.** Chromatographic patterns of papain-treated Fr. II \( \gamma \)-globulin separated on DEAE-cellulose (\( D_{280} = \) optical density at 280 nm). *A,* preparative separation of products; *B,* rechromatography of material from area 1 shown in *A*; *C,* rechromatography of material from area 2 shown in *A*.

In Fig. 7 are shown photographs of the immunoelectrophoretic analyses of chromatographically isolated, papain-treated \( \gamma \)-globulin, compared with unfractionated papain-treated \( \gamma \)-globulin. It can be seen that tube 5 material has a mobility comparable to the S component, while tube 20 material moves with the same mobility as the F component. Cross-absorption experiments showed that peak 5 material completely absorbed the antibodies against the S com-
Figs. 7 a and 7 b. (a) Comparison of immunoelectrophoretic patterns of unfractionated papain-treated γ-globulin (upper patterns), and of two fractions obtained by chromatography (lower patterns). Upper photograph shows pattern of material from tube 5 of chromatogram shown in Fig. 6 A, compared with unfractionated material. Lower photograph shows similar comparison using peak 20 material from chromatogram. Antiserum Rb-137 used in both cases. (b) Unfractionated papain-treated γ-globulin developed with unabsorbed Rb-Be antiserum (central slit), and same antiserum absorbed with peak 5 material (upper slit), and absorbed with peak 20 material (lower slit).
ponent and that tube 20 material similarly absorbed all antibodies against the F component.

Ouchterlony plate analyses showed that tube 5 material and tube 20 material gave single bands, which crossed through each other. The line for tube 5 was undiminished by that for tube 20 when the latter was closest to the antibody reservoir. The line for tube 20 appeared very slightly diminished by that for tube 5.

Fig. 6 shows that the second peak of the chromatography experiment on a preparative scale resolved into two peaks when rechromatographed on columns of analytical dimensions. Experiments with isolated material from peaks II and III showed that they were very closely related immunologically and that

| Sample | $s_{20w}$ in Svedbergs (uncorrected) | Protein concentration | Solvent |
|--------|--------------------------------------|-----------------------|---------|
| Unfractionated papain-treated $\gamma$-globulin (Fr. II) | 3.2* | 7.3 | 0.1 M sodium phosphate pH 7.0, 0.01 M cysteine, 0.002 M versene |
| Peak I | 3.8 | 1.0 | 0.15 M NaCl |
| Peak II | 3.9 | 1.0 | 0.15 M NaCl |
| Peak III | 3.9 | 1.0 | 0.15 M NaCl |

* Main component. This sample contained 8.3 per cent of faster sedimenting material.

the lines for both were completely penetrated by material from peak I in Ouchterlony plate and immuno-electrophoretic analysis. The electrophoretic mobility of peak II material was lower than for peak III and was intermediate between I and III. The uncorrected sedimentation coefficients of material from these peaks are given in Table I.

Chromatographic separation of electrophoretically isolated slowly migrating and fast $\gamma$-globulin that had been treated with papain showed three peaks for each material. Peaks III for both preparations were similar in mobility and immunological specificity. Peaks I differed in mobility in the two samples. For the slowly migrating $\gamma$-globulin peak II was again similar to peak III immunologically, although differing in mobility. For the fast-migrating $\gamma$-globulin, however, peak II contained considerable amounts of partially degraded $\gamma$-globulin and therefore cross-reacted with both peaks I and III. The presence of partially degraded $\gamma$-globulin in the middle peak was observed for two preparations of fast $\gamma$-globulin, but not for slowly migrating $\gamma$-globulin.
nor for the major experiment with purified whole \( \gamma \)-globulin. A small amount of incompletely degraded material could be detected in ultracentrifugal analysis of unseparated papain-treated \( \gamma \)-globulin.

**Fig. 8.** Patterns of papain-split, slowly migrating \( \gamma \)-globulin. Upper pattern developed with antiserum Rb-Sam; lower pattern with antiserum Rb-137. The midcomponent is prominent in this experiment.

**Fig. 9.** Drawing of Ouchterlony plate showing relation between whole \( \gamma \)-globulin and chromatographically isolated products of papain treatment. Well A, whole \( \gamma \)-globulin; well B, tube 5 material; well C, peak 20 material; wells D, antiserum Rb-Sam.

Immunoelectrophoretic analysis of the unfractionated split products of the slowly migrating \( \gamma \)-globulin showed the midcomponent clearly. Fig. 8 shows the split products of slowly migrating \( \gamma \)-globulin with an inflection in the precipitin line where the F component fuses with the midcomponent. In most of the other experiments the midcomponent was obscured by the junction of the lines for the F and S components.

**D. Immunologic Relationship between the Double Precipitation Lines of Whole \( \gamma \)-Globulin and the F and S Components of Papain-Treated \( \gamma \)-Globulin**

A relationship between the double precipitation lines of \( \gamma \)-globulin developed with certain antisera, and the F and S components, was demonstrated by a

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Dr. Jane Morse in this laboratory has studied one antibody, that against insulin, and in preliminary experiments localized it in the S and mid fractions.
Fig. 10. Modified immunoelectrophoretic experiment showing relation between double precipitin line of γ-globulin and S and F components of rabbit-treated γ-globulin. Upper pattern represents whole γ-globulin; lower pattern represents rabbit-treated γ-globulin.
Figs. 11 a and 11 b. Comparison of patterns of double lines produced by unabsorbed antiserum Rb-Sam and by antiserum Rb-Sam absorbed with S component, or F component. The lower portions of a and b show enlargements of the area between the dotted lines. (a) Upper left and lower right slit contain antiserum Rb-Sam. Upper right and lower left slit contain antiserum Rb-Sam absorbed with S component. (b) Upper left and lower right slit contain antiserum Rb-Sam. Lower left and upper right slit contain antiserum Rb-Sam absorbed with F component.
number of methods. Fig. 9 shows an exact drawing of one Ouchterlony plate experiment in which the F and S components isolated chromatographically after treatment with papain were placed in wells on either side of the whole \(\gamma\)-globulin starting material. Antiserum Rb-Sam was then placed opposite each well. Single lines were obtained with the two fragments and a double line with the whole \(\gamma\)-globulin. The outer line of the whole \(\gamma\)-globulin fused with the F component, and the inner line with the S component. Similar results were obtained in numerous experiments which utilized various modifications of the orientation of the Ouchterlony plate wells.

A somewhat more complicated method that gave identical results employed parallel immunoelectrophoresis of whole \(\gamma\)-globulin and the split products (Fig. 10). Between the \(\gamma\)-globulin and the split products, the antiserum well was interrupted. The outer line for whole \(\gamma\)-globulin can be seen to orient itself and fuse with the F component, and the inner line with the S component.

Absorption experiments with F and S components isolated chromatographically also demonstrated the relation of these split products to the double line of whole \(\gamma\)-globulin. This is clearly indicated by the modified immunoelectrophoretic technique illustrated in Fig. 11. Interrupted antiserum slits were again used. In the upper portion of this figure, the top slit contained whole antiserum Rb-Sam on the left, and the same antiserum absorbed with S on the right. The bottom slit contained the antiserum absorbed with S component on the left, and the unabsorbed antiserum on the right. In this experiment complete absorption was achieved and the inner line could be selectively removed with the isolated S fragment. The lower portion of Fig. 11 shows the results of absorption of antiserum Rb-Sam with F component alone, using a similar arrangement of slits. In this case, the absorption is not complete for the outer line and a thin residual line is still visible. It is possible that this represents specific antigenicity of the midcomponent which was not removed by F alone.

**DISCUSSION**

The immunological experiments presented above provide evidence that human \(\gamma\)-globulin has a complex antigenic structure. This complexity, suggested by the observation of a double precipitin line with certain antisera, has become more obvious upon examination of the products after papain treatment of \(\gamma\)-globulin. The double line phenomenon has been clearly related to the two major antigenic split products. Cross-reactions between the S component of these products and the inner precipitin line of whole \(\gamma\)-globulin, and between F component and the outer line, have been consistently observed. Absorption of antisera with either S or F components prevented development of the respective inner or outer whole \(\gamma\)-globulin line. In no case were both lines of whole \(\gamma\)-globulin removed by either S or F components used alone.

These antigenic relations make it highly improbable that double lines are
completely non-specific phenomena resulting from the physico-chemical conditions of precipitation. Many other lines of evidence also seem to make this an unlikely explanation of the present findings. Particular antigen-antibody ratios were not required, and doubling was frequently observed over the entire electrophoretic range of \( \gamma \)-globulin. In many cases, the doubling seemed most obvious at equivalence. In some examples, it was clearly seen at the ends of the precipitin lines. It should be emphasized that only certain antisera produced double lines clearly. Other antisera of similar strength used at comparable dilutions elicited only single lines, although in many instances separation at the ends could be found on careful observation of the immunoelectrophoresis patterns. These observations suggested that the doubling phenomenon was a common one for \( \gamma \)-globulin but that in most instances the lines were so close together that they appeared to be single.

Two alternative explanations can be given for the multiple lines obtained with papain-treated \( \gamma \)-globulin. First they may be attributed to the presence of multiple different antigenic determinants on each \( \gamma \)-globulin molecule, all molecules having similar antigenic structure. The second hypothesis is that certain groups of molecules possess single antigenic determinants different from those of their neighbors. The number of different antigenic types in either case should be at least equal to the number of lines observed.

Support for the multiple determinant model appears in the findings on the myeloma proteins. These proteins, which are unlikely to be heterogeneous, would not be expected to consist of several antigenically different molecules. The patterns obtained with papain-treated \( \gamma \)-myeloma proteins were similar to those of normal \( \gamma \)-globulin, showing two major components which did not cross-react with each other.

Whole myeloma proteins, however, did give double lines in a few instances. If the myeloma proteins are considered to be antigenically homogeneous, this finding must be explained in terms of a single molecular species with two major antigenic groups. In the antigen excess region, antigen may redissolve the precipitate, forming soluble complexes which could diffuse into a region where equivalence was again established, thus forming a second line (12). In antibody excess, the same mechanism may be operative, but only if the rabbit antibodies have properties similar to those of horse precipitins. Rabbit systems in general do not show the formation of soluble complexes in antibody excess. Korngold and van Leeuwen have explained the formation of multiple zones of precipitate by one antigen on the basis of penetration of the precipitate by excess antigen or antibody (13). Jennings (14) has proposed that multiple precipitin zones may be formed by one antigen with several determinants, if excess reactant phenomena occur. Another explanation for double line phenomena was offered by Goudie et al. (15). If it is assumed that antibody to one determinant is precipitating antibody, while the antibody to another determinant is non-precipitating,
a double line may occur by "enhancement;" i.e. insoluble complexes of antigen and non-precipitating antibody are formed with the precipitating antibody.

Richter et al. (16) have described experiments in gel diffusion media, which showed that two haptenic groups on the same carrier molecule form only one precipitin band when tested with a mixture of rabbit antisera specific for each hapten. The single precipitin band corresponded to the hapten-antibody system which reached optimal proportions first. The molecule containing the antigenic groups was immobilized and prevented from reaching optimal proportions with respect to antibody directed against the second hapten. It is known that human serum albumin and its homologous antiserum yielded only one precipitin line in agar, while partially hydrolyzed albumin gave 3 lines with the same antiserum (17). As suggested by Richter et al., these findings may be explained in terms of their model. In contrast to the hypotheses considered above, this model implies that only single precipitin lines are produced by an antigen with multiple determinants, and that double lines are produced by antigenically different molecules.

If one accepts the second hypothesis presented earlier, namely that there are several antigenically different γ-globulins, then a number of structural arrangements can be imagined. Gamma globulin may contain two or more different subfractions each with a single different antigenic determinant group. No independent evidence for the existence of such subspecies has been found in this study. Specimens of γ-globulin of different electrophoretic mobilities produced similar double lines. No antigenic difference between euglobulin and pseudoglobulin, nor between their split products could be detected. Zinc precipitation failed to bring out differences in the double line patterns. Gamma globulin preparations from all persons tested, from individuals of different Grubb groups, as well as from commercial pools, behaved similarly.

Another interpretation that appears to be compatible with the findings is that γ-globulin molecules possess similar antigenic groups but differ in configuration. There may exist γ-globulin molecules that possess both major antigenic groups described in this study, but with one group buried. Thus, although papain treatment would reveal such a group, the whole molecule would have only one determinant on its surface. A population of molecules with some members that had both antigenic determinants available, and some that had only one available, could develop a double precipitin line. An additional possibility is that there may be a double set of molecules with two similar antigenic groups on each molecule one of which is buried in one set, the other of which is buried in the second set. The experimental results do not allow a definite choice to be made among the various hypotheses discussed above.

Partially degraded γ-globulin in a papain-treated sample may complicate the antigenic analysis. Such material could be seen clearly in some of the patterns, notably in those of papain-treated "fast" γ-globulin. This material
appeared in peak II of the chromatographed products. The presence of such a fraction can obscure the interpretation of the crossing of lines attributable to S and F components in immunoelectrophoresis. Until it is certain that such components are absent, questions of minor cross-reactivity between S and F component cannot be resolved. The preponderance of the evidence indicated that the S and F components were antigenically different and that they did not cross-react to any significant degree.

Experiments with slowly migrating and fast migrating γ-globulins have shed light on some structural features of the molecule that determine the electrophoretic range of these subfractions. F components of "slow" and "fast" γ-globulin have similar mobilities. The respective S components, although closely related in antigenic structure, have different mobilities. Not unexpectedly, the S component of "slow" γ-globulin has a lower mobility than the S component of "fast" γ-globulin. These results suggest that the amino acid side chains responsible for antigenic structure differ from those responsible for mobility differences in subfractions of electrophoretically separated γ-globulin.

The question of how many different antigenic determinants can be discerned on γ-globulin has not been fully answered. Varying the times, temperature, and pH of treatment with papain, might yield different patterns, or new groups. The use of enzymes of different specificities may facilitate further analysis. Schultze and Schwick (18), using trypsin and chymotrypsin, digested γ-globulin and analyzed the products by immunoelectrophoresis. The patterns are not clearly identifiable with those obtained in the present study. Several estimates of the minimal number of antigenic determinants of normal γ-globulin have been made (19–21). These estimates have been based on analyses of cross-reactions between myeloma globulins and Bence-Jones proteins and anti-γ-globulin sera. Korngold and Lipari (21) calculated that anti-γ-globulin serum contains at least seven different antibodies that combine with γ-globulin. The present findings support the concept of the multiplicity of antigenic determinants of γ-globulin and provide a direct demonstration of several distinct determinants.

**SUMMARY**

Two major antigenic fragments were obtained from various purified γ-globulin preparations after papain treatment. One, the F component, had a mobility faster than the original γ-globulin and the second, the S component, had a slower mobility. Similar F and S components were also obtained with certain homogeneous myeloma proteins which were closely related to γ-globulin immunologically. Additional minor antigenic components were detected with certain antisera. The technique of immunoelectrophoresis was particularly useful for bringing out the different antigenic constituents obtained after papain treatment.
The F and S components as well as a midfraction were isolated by chromatography on DEAE-cellulose. These were immunologically homogeneous and could be utilized to absorb F and S antibodies from various antisera. The relative amount of F and S antibodies varied in different antisera from individual rabbits immunized with whole γ-globulin.

Whole γ-globulin was separated by zone electrophoresis into a fast migrating and a more slowly migrating fraction. Each of these gave rise to F and S components after splitting with papain. The F components of the two γ-globulins were similar in mobility, while the S components showed marked mobility differences although antigenically they were very similar. The mobility differences of the parent γ-globulin appeared to be primarily related to the differences in the S component.

Certain antisera against pathological γ-globulins were found to give double lines with a wide variety of γ-globulin preparations in agar diffusion. These were shown to be related to the F and S antigenic determinants of γ-globulin. This relationship was evident by a number of procedures utilizing both Ouchterlony plate techniques and immunoelectrophoresis. The question of whether these findings indicate heterogeneity of γ-globulin in relation to the F and S antigenic components, or whether different antigenic groups on one molecule can give rise to separate lines in certain instances, is discussed.

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