Neuman, R. E., and Logan, M. A. (1950), *J. Biol. Chem. 184*, 299.
Ouchterlony, D. (1953), *Acta Pathol. Microbiol. Scand. 32*, 231.
Porter, R. R. (1959), *Biochem. J. 73*, 119.
Porter, R. R. (1962), *in* Basic Problems in Neoplastic Disease, Gellhorn, A., and Hirschberg, E., eds., New York, Columbia University Press, p. 175.
Porter, R. R., and Press, E. M. (1962), *Ann. Rev. Biochem. 31*, 625.
Seifter, S., Gallop, P. M., Klein, L., and Meilman, E. (1959), *J. Biol. Chem. 234*, 285.
Sela, M., and Arnon, R. (1960), *Biochem. J. 75*, 91.
Sela, M., Fuchs, S., and Arnon, R. (1962), *Biochem. J. 85*, 223.
Sela, M., and Katchalski, E. (1955), *J. Am. Chem. Soc. 77*, 3662.
Talmage, D. W., Baker, H. R., and Akeson, W. (1954), *J. Infect. Diseases 94*, 199.

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

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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

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antigen, poly-L-tyrosyl gelatin (pTyrGel),[1] by the peptide-map technique. Fragments obtained by hydrolysis with papain of the normal or immune γ-globulin were reduced in 8 M urea, carboxymethylated, and digested with nagarse (a bacterial proteinase) or pepsin. The peptide mixtures obtained with these enzymes were almost completely soluble in water. Fragments III were also digested in the unreduced form. Two-dimensional separation, by chromatography and high-voltage electrophoresis, of the digest obtained with these two enzymes gave well-resolved peptide patterns.

It was found that the peptide patterns of fragment III derived from antibodies to pTyrGel and from normal rabbit γ-globulin were essentially identical, while the peptide maps of fragments I and II derived from antilysozyme, anti-pTyrGel, and normal rabbit γ-globulin, though very similar, contained some small but reproducible differences. The significance of these findings will be dwelt upon in the discussion.

Experimental

Materials.—Nagarse, batch No. CFF 1072, was obtained from Theikoku Chemical Industry, Osaka, Japan. Pepsin (twice crystallized), chymotrypsin (twice crystallized), trypsin (twice crystallized, salt free), and papain (twice crystallized) were obtained from Worthington. Water-insoluble papain was prepared as described by Cebra *et al.* (1961). DEAE-cellulose was obtained from Eastman Kodak, CM-cellulose (0.7 meq/g) was obtained from Bio-Rad Laboratories, and Sephadex was obtained from Pharmacia, Uppsala, Sweden. Urea (Analar) and mercaptoethanol (Fluka, Switzerland) were used without further purification. Iodoacetic acid (Eastman Kodak) was recrystallized from ether–petroleum ether.

Preparation and Fragmentation of Rabbit γ-Globulin and Antibodies.—Rabbit γ-globulin was prepared from pooled rabbit serum by precipitation at 0.4 ammonium sulfate saturation at 4° and subsequent chromatography on a DEAE-cellulose column under the conditions described by Levy and Sober (1960). Antibodies to lysozyme were purified immunospecifically according to Givol *et al.* (1962). Preparative amounts of these antibodies were purified from batches of 40 ml of a high-titer-pooled antiserum (2–3 mg antibodies/ml) by passing the dissolved immunological precipitate (*p*H 1.8) through a Sephadex G-100 column (60 × 3 cm, i.d.). Antibodies to pTyrGel were isolated by the procedure which utilizes selective proteolysis of the immunological precipitate with collagenase, and still contained ^{131}I-labeled pieces of the antigen bound to the combining sites of the antibody (Givol and Sela, 1964).

The fragmentation of the above γ-globulin or antibodies was performed with water-insoluble papain followed by thioglycolate (Cebra *et al.*, 1961; Givol and Sela, 1964). Fragment III crystallized out from the reaction mixture after standing for 12 hours at 4°. The crystals were centrifuged, washed with 0.1 M phosphate buffer, *p*H 7.0, dissolved in 0.05 M acetic acid and, after dialysis against 0.02 M acetic acid, were lyophilized. Only the crystalline portion of fragment III was used in this work. Fragments I and II were separated on a CM-cellulose column (Porter, 1959) and, after dialysis against 0.02 M acetic acid, were lyophilized.

[1] Abbreviations used in this work: pTyrGel, poly-L-tyrosyl gelatin; DEAE, diethylaminoethyl-; CM, carboxymethyl-.

Reduction and Carboxymethylation of the Fragment.—Reduction and alkylation of the disulfide bridges was carried out essentially according to Sela *et al.* (1959), except that mercaptoethanol was used for the reduction instead of thioglycolate (Anfinsen and Haber, 1961). In a typical experiment 50 mg of γ-globulin or antibody fragment was dissolved in 4.5 ml of 9 M urea and the *p*H of the solution was adjusted to *p*H 8.5 by the addition of 5% trimethylamine. To this solution mercaptoethanol (0.5 ml) was added and nitrogen was flushed in for 5 minutes. The reaction mixture was allowed to stand at room temperature for 4 hours and then the reduced protein was precipitated by addition of 30 ml of ice-cold acid acetone (acetone–1 N HCl, 39:1). After centrifugation and washing with the cold acid acetone, the precipitate was dissolved in 8 M urea (5 ml, previously adjusted to *p*H 3 with HCl in order to prevent oxidation of the sulfhydryl groups), and a concentrated solution of iodoacetate (containing 0.5 g iodoacetic acid brought to *p*H 8.2 with NaOH) was added. The alkylation was performed in a *p*H-stat and the *p*H was maintained at 8.2 by the addition of 0.1 N NaOH. The reaction was completed within 1–2 hours and the alkylated product was dialyzed exhaustively against water. During dialysis a small amount of material precipitated; nevertheless the whole content of the dialysis bag was lyophilized (80% recovery).

Proteolytic Digestion of the Fragments.—Digestions with nagarse, trypsin, or chymotrypsin were performed in 0.1 M ammonium carbonate, *p*H 8.0, for 24 hours, with a 1:50 ratio of enzyme to substrate. The digestion with pepsin was carried out with the same enzyme-substrate ratio, in a solution adjusted to *p*H 2.0 by 0.1 N HCl, for 24 hours. The extent of digestion was routinely examined by a two-dimensional separation of the peptides formed by chromatography and high-voltage electrophoresis.

The unreduced fragments were relatively resistant to hydrolysis by trypsin, chymotrypsin, and nagarse, as was revealed by the small number of peptides appearing after chromatography and electrophoresis of the digest. Heating of the fragment for 10 minutes at 100° before digestion did not improve the results appreciably. Upon proteolysis with pepsin, the digestion of unreduced crystalline fragment III yielded a well-resolved peptide pattern, while the digests of fragments I and II contained some undigested material which did not migrate from the origin upon chromatography and electrophoresis.

Digestion by the above enzymes of reduced and alkylated fragments yielded, upon chromatography and electrophoresis, better peptide patterns than those obtained from digests of unreduced fragments. Nevertheless, after the digestion with trypsin or chymotrypsin there still remained an undigested core, which was almost absent after hydrolysis with pepsin or nagarse. It was decided, therefore, to use these latter two enzymes in the digestion of reduced and alkylated fragments for the comparison of the peptide patterns of the digests. The reduced and alkylated fragments I, II, and III of rabbit γ-globulin were compared in this manner, as well as fragments I and II of the purified antibodies to lysozyme and to pTyrGel. The peptide patterns of the peptic digests of unreduced crystalline fragment III from rabbit γ-globulin and from anti-pTyrGel were also compared.

For hydrolysis with pepsin the γ-globulin or antibody fragment (10 mg) was suspended in water (0.7 ml) and 0.1 N HCl was added (about 0.15 ml) to bring the solution to *p*H 2. Under these conditions part of the material did not dissolve. A pepsin solution

(2 mg/ml in 0.01 N HCl) was prepared before use and 0.1 ml of it was added to the fragment suspension. After 30–60 minutes at 37° the solution became clear, but after 7–8 more hours some precipitate had formed. The solution was allowed to stand at 37° for 24 hours, and then it was centrifuged and the supernatant (containing 85% of the original weight) was dried *in vacuo* over NaOH and P_2O_5.

For the hydrolysis with nagarse the fragment (10 mg) was dissolved in 0.1 M ammonium carbonate buffer, *p*H 8.0 (0.9 ml), and 0.1 ml of nagarse solution (2 mg/ml in the same buffer) was added.

After 24 hours at 37° practically no precipitate had formed. The solution was dried *in vacuo* over P_2O_5 and NaOH.

Two-dimensional Separation of Peptides.—Peptide patterns of the various digests were obtained by a two-dimensional paper chromatography–paper electrophoresis technique similar to that described by Katz *et al.* (1959). The dried digests were dissolved in water to a final concentration of 50 mg/ml and applied (1 mg in 20 μl) to Whatman No. 3 paper (57 × 46 cm). The point of application was 9 cm from the top and 15 cm from the left edge of the paper. The peptides were subjected to descending chromatography in the organic phase of a freshly prepared butanol–acetic acid–water (25:6:25) mixture for 11 hours. The chromatogram was dried in air at 20° for at least 90 minutes before electrophoresis. The dry chromatogram was then wetted by spraying with a buffer composed of pyridine–acetic acid–water (1:10:189), *p*H 3.5. The buffer fronts were allowed to coalesce along the line of the origin and the chromatogram was then placed in a Lucite tank similar to that described by Katz *et al.* (1959). Electrophoresis was carried out at 40 v/cm and 120–150 ma for 50 minutes. The paper was dried at 60° and heated for 10 minutes to 100°. The peptides were located by dipping the paper in 0.5% ninhydrin in acetone and heating at 60° for 20 minutes. Peptide patterns were photographed immediately, using a green or yellow-green filter. Reducing sugars were located according to Trevelyan *et al.* (1950) on paper that had not been developed with ninhydrin.

Sedimentation Analyses.—Measurements were carried out in a Spinco Model E ultracentrifuge at 56,100 rpm.

Results

Preparation of Fragments from γ-Globulin and Antibodies.—Fragments I, II, and the crystalline fragment III of rabbit γ-globulin were prepared as described under Experimental. Sedimentation analyses were carried out routinely on the products at the various stages of fragmentation. Only 70–80% of fragment III was crystallized out under the conditions used (12 hours at 4°). The total amounts obtained in several fractionations were 841 mg, 867 mg, and 797 mg of fragments I, II, and III (crystals), respectively. The remainder of fragment III, which did not crystallize from the papain digest, was separated from fragments I and II on the CM-cellulose.

The fragments of antibodies to pTyrGel (53 mg, 55 mg, and 48 mg, respectively for fragments I, II, and crystalline fragment III) were prepared from antibodies isolated by the procedure which makes use of collagenase to solubilize the immunological precipitate (Givol and Sela, 1964). When ^{131}I-labeled pTyrGel was used to precipitate the antibodies it was possible to follow the contamination of the antibody fragments by radioactive pieces of the antigen. While crystalline fragment III was practically free of such antigen peptides, fragments I and II still had peptides of the antigen attached to them (Givol and Sela, 1964). Upon reduction in 8 M urea (see Experimental) the labeled peptides of the antigen were released completely from the antibody fragments, as apparent from the observation that after precipitation with acid acetone the reduced fragments contained no radioactivity. Similar results were obtained by passing the solution of the reduced fragments through a Sephadex G-50 column which was equilibrated with 0.1 M acetic acid in 4 M urea. In this case, too, the protein fraction was clearly separated from the radioactive materials. Thus, the reduced fragments obtained were free from contamination with peptides of the antigen, and this finding indicates that, under the conditions of reduction in 8 M urea, the antibody fragment loses its ability to bind the antigen.

The fragments of antibodies to lysozyme were prepared from combined batches of antibodies that were purified from various pools of antilysozyme serum under acid conditions (Givol *et al.*, 1962). While the sedimentation constant after fragmentation of this antibody was 3.5 S, similar to that of the fragments obtained by the same procedure from normal γ-globulin and antibodies to pTyrGel, it was not possible in this case to crystallize out fragment III, even after 48 hours in the cold. Upon chromatography on a CM-cellulose column fragments I and II were eluted as usual, while only a small amount of fragment III was recovered. The total amount of fragments obtained from the column was 73 mg, 66 mg, and 17 mg for fragments I, II, and III, respectively. Because of the lack of crystalline fragment III and the low recovery of this fragment from antibodies to lysozyme, its analysis by the peptide-map technique was not attempted. Similar difficulties in the crystallization and recovery of fragment III were observed also with normal rabbit γ-globulin after exposure for 3 hours to an acid solution of *p*H 1.8, under which conditions isolation of antibodies to lysozyme is performed.

Peptide Maps of Rabbit γ-Globulin Fragments.—Fragments I, II, III (crystalline) were reduced and alkylated in the presence of 8 M urea. No difficulties of the kind described by Markus *et al.* (1962) were observed at the stage of acid acetone precipitation of the reduced fragment III, using acid ethanol for the precipitation of the reduced fragment. The recovery of each of the three fragments was around 80%.

The reduced and alkylated fragments were subjected to hydrolysis with pepsin or nagarse. Preliminary separation of the peptides from the digests showed that 11 hours of chromatography in the first dimension and 50 minutes of high-voltage electrophoresis in the second dimension are adequate for resolution of the peptides in each direction Figure 1 shows the peptide pattern obtained from peptic or nagarse digests of the three reduced and alkylated fragments. Around 50–60 peptides were resolved in the peptide pattern obtained from nagarse digests of each fragment, and 40–50 peptides in the peptide patterns obtained from the peptic digests. The peptide patterns obtained from fragment III after hydrolysis with either of these two enzymes showed a much better resolution than those obtained from the digests of fragments I or II. The last two always contained regions of smears and unresolved peptides. The main conclusion that can be drawn from Figure 1 is that the peptide patterns of fragment III are very different from those of fragment I or II, while the peptide patterns of these two are very similar. Nevertheless, it is possible to detect some reproducible differences between the peptide patterns

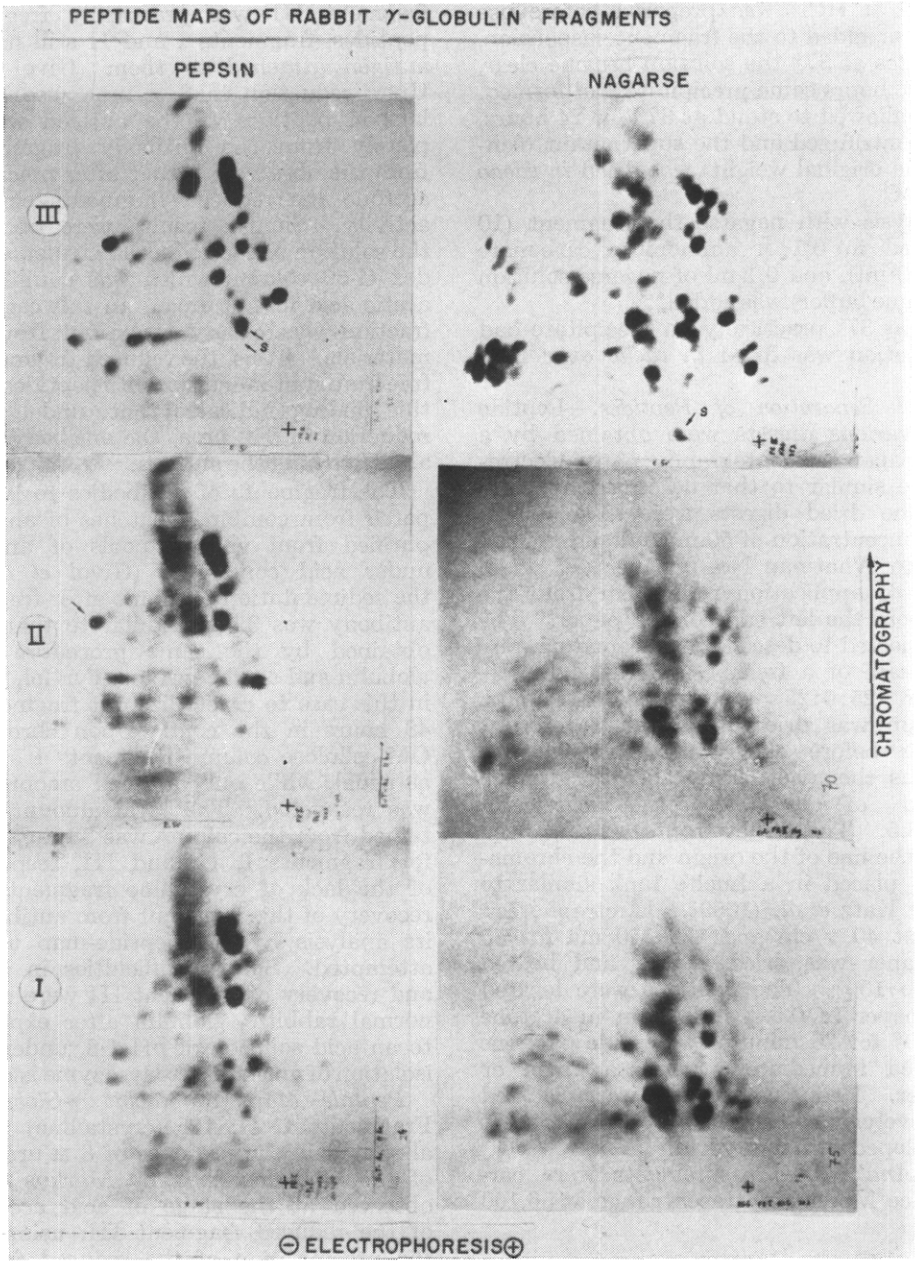

FIG. 1.—Peptide patterns of reduced and alkylated fragments (I, II, and III) of rabbit γ-globulin from normal sera. The patterns on the left-hand column are of pepsin hydrolysates and those on the right-hand column are of nagarse hydrolysates. The point of application is marked by a cross in each pattern. Separation of the peptides was performed by chromatography in the first dimension and electrophoresis in the second dimension. The arrows in the pattern of fragment III (marked by *s*) show the place where reducing sugar was detected. The arrow in the pepsin-peptide pattern of fragment II (left column, middle picture) shows the positively charged peptide which is absent in the pattern of fragment I.

of fragments I and II. These differences are more pronounced in the peptide patterns obtained from the peptic hydrolysates. Especially noticeable in the peptic digest is one positively charged peptide which is present in the pattern of fragment II (arrow) but not in that of fragment I. This peptide may be partially responsible for the differences in the adsorption of fragments I and II on CM-cellulose, which permit their separation on this cation exchanger.

When papers on which two-dimensional separation of the peptides was performed were stained and developed with a reagent for the detection of reducing sugars (Trevelyan *et al.*, 1950), only fragment III showed positive results. One positive spot was detected in the product of the digestion of fragment III with nagarse, while two neighboring positive spots were found in the product of the digestion with pepsin (Fig. 1, arrows).

Peptide Maps of Antibody Fragments.—Since fragment III in the native form was hydrolyzed very readily by pepsin and the resolution in the peptide pattern of the digest was good, unreduced fragment III from antibodies to pTyrGel and from normal γ-globulin was digested by pepsin, and the digests were subjected to a two-dimensional separation. The peptide patterns obtained are shown in Figure 2. Evidently, all the peptides present in the peptide map of fragment III derived from γ-globulin have their counterparts in the peptide map of fragment III derived from the antibody, although some of the peptides seem to appear as rela-

tively stronger spots on the peptide map of the fragment III from the antibody.

The peptide patterns obtained from the digests of reduced and alkylated fragments I and fragments II by nagarse are shown in Figure 3. Considering the differences in the procedures used in the course of preparation of the γ-globulin, the antibodies to lysozyme and the antibodies to pTyrGel from which these fragments were derived, the similarity in their peptide patterns is remarkable. Some small differences that were observed to exist between the peptide patterns of fragments I and II from normal γ-globulin persist also when these two fragments are compared in the case of purified antibodies. It is also possible to conclude that the number of peptides in the patterns from antibody fragments is essentially neither larger nor smaller than the number of peptides present in the equivalent peptide maps for normal γ-globulin fragments. Areas of smears in the peptide maps of γ-globulin fragments were not resolved to more distinct peptide spots in the patterns obtained from fragments of antibodies. Nevertheless, it is possible to detect at least three peptides in the peptide pattern obtained from fragment I of antibodies to lysozyme which are not present in the peptide pattern obtained from antibodies to pTyrGel. One of these peptides is present in the peptide pattern of fragment I obtained from γ-globulin, and the others are not. These differing peptides do not have their counterparts in the peptide patterns of fragments II, obtained from the antibodies and γ-globulin, but other small differences are present also there (see arrows).

Figure 4 shows the peptide patterns of peptic digests of reduced and alkylated fragments I and fragments II. The comparison of these patterns reveals more differences between the fragments obtained from the two different antibodies, as well as between each of them and the fragments of γ-globulin, than were observed in the corresponding patterns obtained after nagarse digestion.

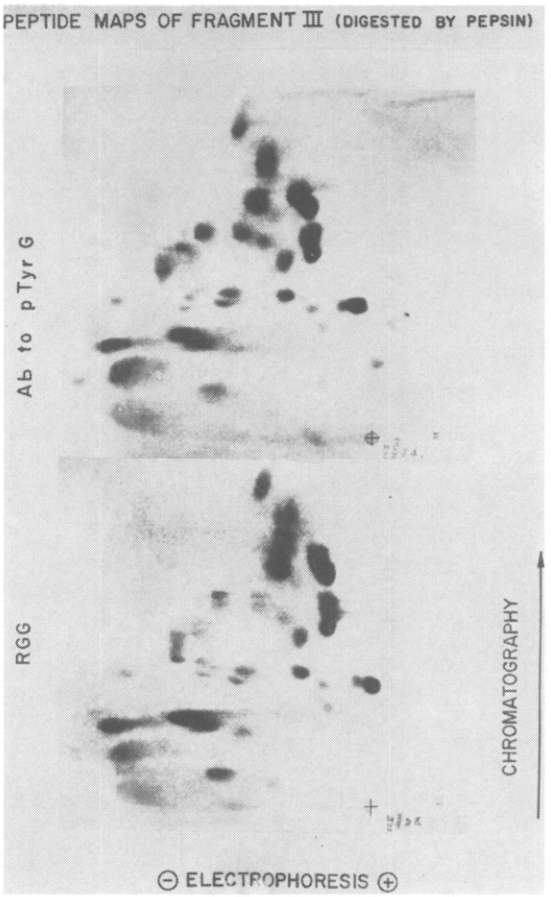

FIG. 2.—Pepsin-peptide patterns of fragment III from rabbit γ-globulin and antibodies to pTyrGel. The point of application is marked by a cross in each pattern. RGG, rabbit γ-globulin; Ab, antibody; pTyrG, poly-L-tyrosyl gelatin.

DISCUSSION

The ease of detection by the peptide-map technique of small differences in the covalent structure of proteins depends primarily on the size of the protein molecule. For this reason we decided in the study reported here to compare enzymatic digests of fragments derived from normal γ-globulin or antibodies, rather than digests of the intact γ-globulins. In this study we have used as the starting materials the fragments obtained by the action of papain and thioglycolate on γ-globulins. Of the three resulting types of fragments (I, II, and III), fragments I and II still possess the full capacity to react with antigens (Porter, 1959; Nisonoff and Woernley, 1959), which implies that the antibody-combining site remains intact. Using a short digestion with water-insoluble papain as a first stage in this fragmentation, only a minimal number of peptide bonds was split (Cebra *et al.*, 1961).

In order to obtain reproducible peptide maps the opening of the disulfide bridges in the γ-globulin fragments before proteolysis seemed desirable. Reduction and alkylation are preferable to oxidative cleavage of disulfide bridges in proteins containing tryptophan (Sela *et al.*, 1959). The fragments obtained by treatment of γ-globulin with papain and reduction were therefore further reduced in 8 M urea and the sulfhydryl groups formed were alkylated with iodoacetate.

The enzymes used in this study for the digestion of the γ-globulin fragments were nagarse and pepsin, as the action of these two enzymes yielded peptide mixtures which were completely water soluble in the case of nagarse and soluble to the extent of 85% in the case of pepsin. In our experiments chromatography preceded high-voltage electrophoresis, as it was found that these conditions permitted the best resolution of the peptides in the digests.

It may be concluded from a comparison of fragments I, II, and III derived from a normal rabbit γ-globulin preparation (Fig. 1) that the peptide map of III is very different from those of I and II, while the last two yield peptide maps which are very similar to each other but contain significant and reproducible differences. These results are in agreement with those reported by Gitlin and Merler (1961b) on a comparison of peptide maps of chymotrypsin or subtilisin digests of performic acid–oxidized fragments I, II, and III, and with the autoradiographic data of Roholt *et al.* (1962) on peptic digests of unreduced fragments I and II. The presence of a strongly basic peptide in the two-dimensional map of the peptic digest of fragment II, which is absent in the corresponding peptide map of fragment I, is compatible with the order of elution of fragments I and II upon ion-exchange chromatography on CM-cellulose, as well as with the elution of γ-globulins containing fragments I or fragments II on CM-cellulose (Palmer *et al.*, 1962) or on DEAE-Sephadex (Sela *et al.*, 1963b).

An additional difference between fragments I and II and fragment III is apparent from the selective staining of their peptide maps for reducing sugars. Only the peptide maps of fragment III contained spots which gave a positive reaction for sugar.

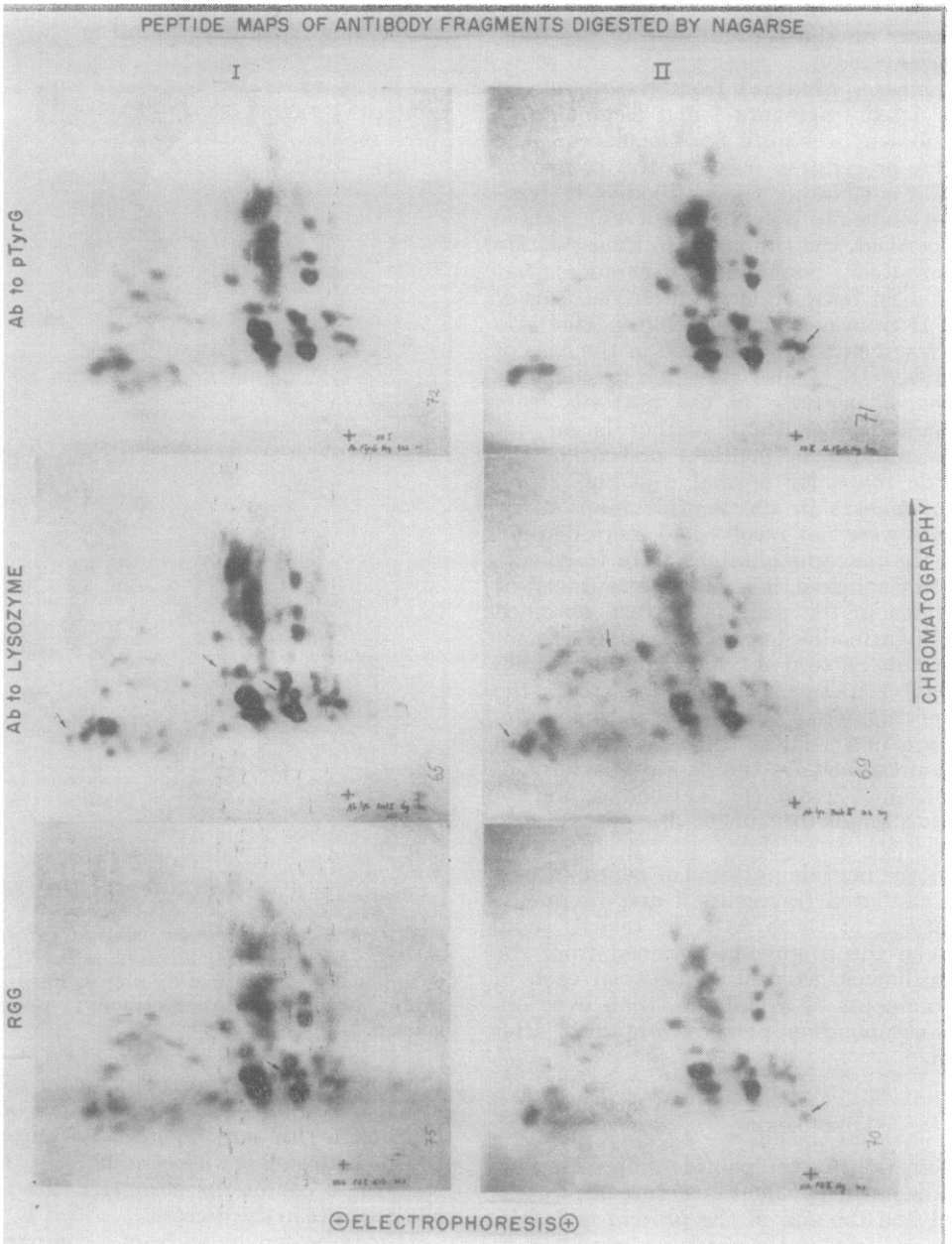

FIG. 3.—Nagarse-peptide patterns of reduced and alkylated fragments from rabbit γ-globulin and antibodies. The patterns on the left column are of fragment I and those on the right column are of fragment II. Areas indicated by arrows show differences between the patterns. RGG, rabbit γ-globulin; Ab, antibody; pTyrG, poly-L-tyrosyl gelatin.

In view of the ease of digestion of fragment III by pepsin, samples of fragment III derived from normal γ-globulin and from antibodies to pTyrGel were compared by the peptide-map technique in the unreduced form (Fig. 2). The fragment III derived from antibodies to lysozyme was not subjected to digestion, as it was not obtained in the crystalline form. Apparently the exposure of the antibodies to acid conditions during the purification, while not affecting significantly the antibody-combining sites, has a definite effect on that portion of the γ-globulin molecule which yields fragment III after hydrolysis with papain.

From a comparison of the peptide maps of fragments III (Fig. 2) it may be concluded that the patterns are essentially identical, implying that the fragments themselves, derived either from normal rabbit γ-globulin or from rabbit antibodies to pTyrGel, are identical. A different conclusion was reached by Gitlin and Merler (1961a), who observed small but reproducible differences between peptide maps of digests of performic acid–oxidized fragments III derived from antibodies to different pneumococcal polysaccharides. On the other hand, in a recent preliminary report Nelson *et al.* (1963) reach the conclusion, similar to ours, that samples of fragments III derived from two different antibodies and from normal γ-globulin are virtually identical.

If the assumption is made that the sequence of peptide segments forming the combining sites differs from one antibody to another, and that the so-called "normal" γ-globulin is mainly a mixture of antibodies to a variety of unknown antigens, it would be expected that peptide maps derived from fragments I and II of immunospecifically purified antibodies should show a greater number of distinct detectable spots than those derived from "normal" γ-globulins, as the spots originating from the many chemically different combining sites would be, in the last case, below the

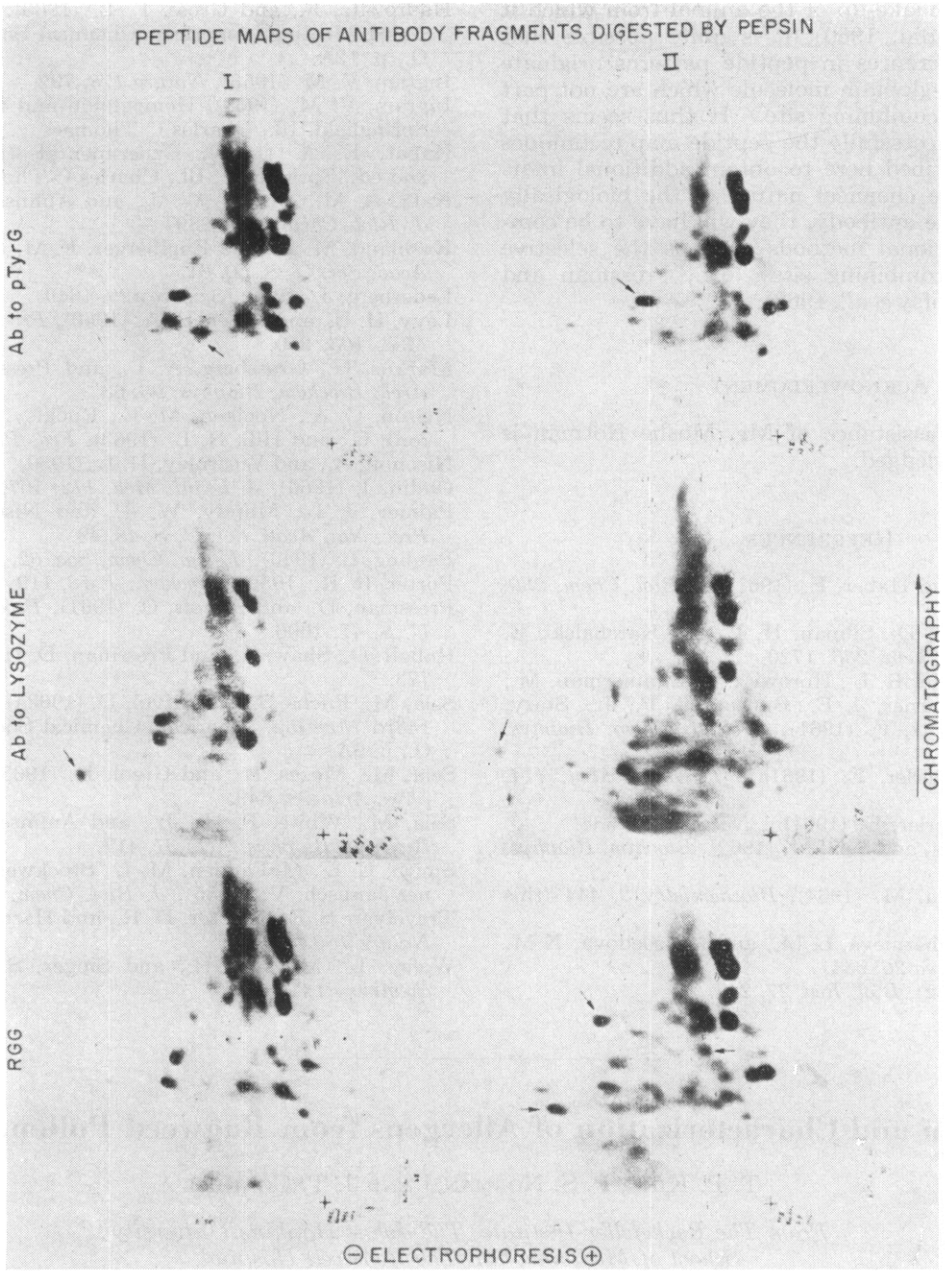

FIG. 4.—Pepsin-peptide patterns of reduced and alkylated fragments from rabbit γ-globulin and antibodies. The patterns on the left column are of fragment I and those on the right column are of fragment II. Areas indicated by arrows show differences between the patterns. RGG, rabbit γ-globulin; Ab, antibody; pTyrG, poly-L-tyrosyl gelatin.

threshold of detection. It is seen clearly in Figures 3 and 4 that this is not the case, as the number of distinct peptides in the patterns of fragments I and II derived from antibodies to lysozyme, antibodies to pTyrGel, and normal γ-globulin is almost identical. Thus, while there are some peptides in the pattern derived from fragment I of antibodies to lysozyme which are absent in the pattern derived from normal γ-globulin, there are also peptides in the pattern of the fragment I of normal γ-globulin which are missing in that of antibodies to pTyrGel. A similar situation obtains for fragments II.

The main conclusions to be drawn from a comparison of fragment I or II of the antibodies and of the normal γ-globulin is the remarkable similarity between the peptide patterns, even though, in contrast to fragments III, small but reproducible differences are observed both between one antibody and another and between the antibodies and the normal γ-globulin. This is in agreement with the findings of Gitlin and Merler (1961a) from studies of antibodies to pneumococcal polysaccharides and normal rabbit γ-globulin. On the other hand, Haurowitz and Gross (1963) have reported recently no differences between the peptide patterns of tryptic digests of fragments from antibodies to phenylarsonate and phenyltrimethylammonium haptens, even though they observed differences between the peptide patterns of these antibodies and normal γ-globulin. Differences between electrophoretic patterns of a normal and an immune rabbit γ-globulin were reported also by Roholt *et al.* (1962).

If the small differences in the peptide maps are due to differences in the combining sites of the antibodies, one would have to conclude that such combining sites may form only a small part of the antibody molecule. Nevertheless, as this study was carried out with antisera pooled from several rabbits, and in view of the known variation in rabbit γ-globulin in accordance

with the genetic make-up of the animal from which it was derived (Oudin, 1960), it is quite possible that the reported differences in peptide patterns originate in areas of the γ-globulin molecule which are not part of the antibody-combining site. It thus seems that in order to use successfully the peptide-map techniques of the type described here to obtain additional information about the chemical nature of the biologically active areas of the antibody, they will have to be combined with additional methods such as the selective tagging of the combining sites (e.g., Pressman and Roholt, 1961; Wofsy *et al.*, 1962).

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REFERENCES

Anfinsen, C. B., and Haber, E. (1961), *J. Biol. Chem. 236*, 1361.
Cebra, J. J., Givol, D., Silman, H. I., and Katchalski, E. (1961), *J. Biol. Chem. 236*, 1720.
Fleischer, S., Hardin, R. L., Horowitz, J., Zimmerman, M., Gresham, E., Turner, J. E., Burnett, J. P., Jr., Stary, Z., and Haurowitz, F. (1961), *Arch. Biochem. Biophys. 92*, 329.
Gitlin, D., and Merler, E. (1961a), *J. Exptl. Med. 114*, 217.
Gitlin, D., and Merler, E. (1961b), *Nature 190*, 634.
Givol, D., Fuchs, S., and Sela, M. (1962), *Biochim. Biophys. Acta 63*, 222.
Givol, D., and Sela, M. (1964), *Biochemistry 3*, 444 (this issue).
Gurvich, A. E., Gubernieva, L. M., and Miasoedova, K.M. (1961), *Biokhimiya 26*, 934.
Haurowitz, F. (1952), *Biol. Rev. 27*, 247.
Haurowitz, F., and Gross, F. L. (1963), Abstracts of the 143rd Meeting, American Chemical Society, Cincinnati, O., p. 12A.
Ingram, V. M. (1956), *Nature 178*, 792.
Ingram, V. M. (1961), Hemoglobin and its Abnormalities, Springfield, Ill., Charles C Thomas.
Kabat, E. A. (1961), Experimental Immunochemistry, 2nd ed., Springfield, Ill., Charles C Thomas, p. 241.
Katz, A. M., Dreyer, W. J., and Anfinsen, C. B. (1959), *J. Biol. Chem. 234*, 2897.
Koshland, M. E., and Englberger, F. M. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 61.
Lederberg, J. (1959), *Science 129*, 1649.
Levy, H. B., and Sober, H. A. (1960), *Proc. Soc. Exptl. Biol. Med. 103*, 250.
Markus, G., Grossberg, A. L., and Pressman, D. (1962), *Arch. Biochem. Biophys. 96*, 63.
Nelson, C. A., Noelken, M. E., Buckley, C. E., III, Tanford, C., and Hill, R. L. (1963), *Fed. Proc. 22*, 657.
Nisonoff, A., and Woernley, D. L. (1959), *Nature 183*, 1325.
Oudin, J. (1960), *J. Exptl. Med. 112*, 107, 125.
Palmer, J. L., Mandy, W. J., and Nisonoff, A. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 49.
Pauling, L. (1940), *J. Am. Chem. Soc. 62*, 2643.
Porter, R. R. (1959), *Biochem. J. 73*, 119.
Pressman, D., and Roholt, O. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1606.
Roholt, O., Shaw, A., and Pressman, D. (1962), *Nature 196*, 773.
Sela, M., Fuchs, S., and Givol, D. (1963a), Abstracts of the 143rd Meeting, American Chemical Society, Cincinnati, O., p. 9A.
Sela, M., Mozes, E., and Givol, D. (1963b), *Biochim. Biophys. Acta 78*, 649.
Sela, M., White, F. H., Jr., and Anfinsen, C. B. (1959), *Biochim. Biophys. Acta 31*, 417.
Smith, E. L., McFadden, M. L., Stockwell, A., and Buettner-Janusch, V. (1955), *J. Biol. Chem. 214*, 197.
Trevelyan, S. E., Procter, D. R., and Harrison, J. S. (1950), *Nature 166*, 444.
Wofsy, L., Metzger, H., and Singer, S. J. (1962), *Biochemistry 1*, 1031.

Isolation and Characterization of Allergens from Ragweed Pollen. II*

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The predominant allergen from ragweed pollen has been characterized as a protein of 37,800 molecular weight by physicochemical and immunochemical procedures. The active protein constitutes about 6% of the proteins in the pollen extract and it has been designated as antigen E. Antigen E can be isolated in four chemical forms. These forms have identical molecular weights and amino acid compositions but they differ in their charges. On removal of antigen E from pollen extract by precipitation with specific rabbit antiserum, a 40-fold decrease of the allergenic activity was observed by direct skin tests of the supernatant on eight sensitive patients. The finding suggests that antigen E represents at least 90% of the allergenic activity in ragweed pollen.

We have previously reported on the isolation of a highly allergenic protein fraction from ragweed pollen (King and Norman, 1962). The active fraction was designated as IV and was isolated from pollen extracts by ammonium sulfate precipitation followed with DEAE-cellulose[1] and Sephadex chromatography. Immunodiffusion studies of fraction IV with rabbit anti-ragweed sera showed that it contained a principal component which was called antigen E. However

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[1] Abbreviations used in this work: DEAE, diethylaminoethyl-; TEAE, triethylaminoethyl-; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate.