Comparison of the Tryptic Peptides from Rabbit γ-Globulin and Two Specific Rabbit Antibodies*

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ABSTRACT: The tryptic peptides from *S*-carboxymethyl or S-carboxamidomethyl chains and fragments of rabbit γ-globulin have been compared by two different peptide-mapping techniques. The nature of the peptide maps offers additional support for the four-chained structure of γ-globulin and specific antibodies, and for the heterogeneity of the lighter B chains from γ-globulin. Peptide maps of homologous chains or fragments from nonspecific γ-globulin and from two specific antibodies are remarkably similar, and no direct evidence for significant differences in amino acid sequence among them could be detected. Sufficient peptides were not recovered from that portion of the A chain, which forms a part of fragment I, to account for the entire structure of that part of the molecule. Sequence differences associated with antibody specificity could therefore reside in that part of the molecule, but such an interpretation is at present entirely speculative since the tryptic digest of A chains and fragment I contained insoluble material which could not be analyzed.

In order to understand antibody specificity it is essential to obtain a detailed knowledge of the structure of γ-globulin. An examination of the tryptic peptides of this protein represents a step toward that goal. In particular it can provide evidence concerning the following aspects of the structure.

The Four-Chain Model. Recent work indicates that γ-globulin consists of four polypeptide chains linked by disulfide bonds: two identical A chains with molecular weight near 50,000, and two identical B chains with molecular weight near 25,000 (Fleischman *et al.*, 1963; Edelman and Gally, 1964; Marler *et al.*, 1964). The molecule can be separated into these chains by reduction and chromatography. Subdivision of the protein can be achieved also by the action of papain (Porter, 1959), which yields two identical fragments which carry the antibody-combining sites and a third fragment which has no antibody activity. Each fragment has a molecular weight near 50,000. The active fragments derived from rabbit γ-globulin are called fragment I or II, depending on the electrophoretic mobility of the γ-globulin from which they are derived (Palmer *et al.*, 1962). The inactive fragment is called fragment III. The papain split is believed to occur near the center of the A chain, so that fragment I or II should consist of a B chain plus about half an A chain. Fragment III should consist of two identical chains, each of which is that portion of the A chain which does not appear in fragment I or II. It is evident that this model can be tested by examining separately the tryptic peptides of the A and B chains and of the fragments derived from papain cleavage, since most peptides should be observed to occur twice: once in the hydrolysate of a polypeptide chain and once in the hydrolysate of one of the fragments.

Chemical Basis for Antibody Specificity. The problem of the chemical basis of antibody specificity can be approached by comparing tryptic peptides from different specific antibodies with each other and with peptides derived from nonspecific γ-globulin. It was believed at one time that noncovalent interactions were primarily responsible for conferring specifically reactive sites upon an antibody molecule (Pauling, 1940). If that were so, then no appreciable differences between the peptides of different antibodies and nonspecific γ-globulin would be expected. Persuasive evidence now exists, however, which indicates that antibody specificity results from specificity in amino acid sequence (Buckley *et al.*, 1963; Haber, 1964; Whitney and Tanford, 1965). This should be reflected in differences in tryptic peptides. However, the differences need occur only in fragment I or II of the protein, since fragment III does not carry or apparently influence the active site. Moreover, the striking similarities in the chemical properties and the amino acid composition (Koshland and Englberger, 1963) of different antibodies suggest that differences between them may be confined to only a small portion of fragment I or II. If this is so, substantial portions can still have a unique amino acid sequence common to all antibodies.

Microheterogeneity of γ-Globulin. A major aspect of the chemistry of γ-globulin, which has hampered progress in the elucidation of its structure, is the fact that physically and chemically homogeneous preparations cannot be obtained. Two sources of microheterogeneity are present: genetic variations which are unrelated to antibody specificity (Feinstein *et al.*, 1963),

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and variations associated with antibody specificity. The latter kind of heterogeneity apparently cannot be avoided even if a highly purified antibody is used. Eisen and Siskind (1964) and Day *et al.* (1963) have shown that purified antibodies consist of mixtures of molecules differing in their affinity for antigen. Both kinds of microheterogeneity are likely to be reflected by the appearance in the tryptic hydrolysate of a large number of different peptides (in excess of the number predicted on the basis of the number of trypsin-susceptible peptide bonds), each in low yield. If the number of different microheterogeneous species is sufficiently large, the yield of some individual peptides with unique sequence may be so small as to make them undetectable.

The work described in this paper was undertaken with these problems in mind. The results are of a preliminary and qualitative nature in that individual peptides have been identified so far only in terms of their location on a two-dimensional paper chromatogram, or in terms of their position in the eluate from a chromatographic column. The results are nevertheless of considerable significance, and provide new information concerning the problems cited here. In particular, they suggest that sources of heterogeneity may be confined to relatively small portions of the molecule, and that much of the structure of γ-globulin may have a unique amino acid sequence.

Experimental

Preparation of γ-Globulin. Three different preparations of nonspecific rabbit γ-globulin were used in these studies. One was prepared from pooled rabbit serum (Pel-Freez Biologicals, Rogers, Ark.) by fractionation with sodium sulfate according to the method of Kekwick (1940). The yield of γ-globulin was about 3 g/liter of serum, representing only one-third of the total γ-globulin content of serum. The γ-globulin obtained in this manner was not grossly heterogeneous on ultracentrifugal or electrophoretic analysis. Ninety % of the preparation had an $s_{20,w}$ of 6.7 S, whereas the remainder of the protein had an $s_{20,w}$ of 10 S. Electrophoresis on starch gels at *p*H 8.6 (Smithies, 1959) revealed only traces of β-globulins and no detectable amounts of other serum proteins. Immunoelectrophoresis revealed several α- or β-globulin components, as contaminants, but in amounts which were insignificant. This preparation was the only source of A and B chains used in this study. It is noteworthy that this preparation gave about ten times as much fragment I as fragment II. A second preparation of nonspecific γ-globulin was obtained from Pentex Biochemicals, Kankakee, Ill. When this material, prepared by fractionation with ethanol, was examined by electrophoresis and ultracentrifugation, it was not as pure as γ-globulin obtained by salt fractionation. However, since this preparation was used only for the preparation of fragments I and III, and the fragments from this material were indistinguishable from fragments prepared from less heterogeneous γ-globulin, the impurity of this preparation did not alter significantly the results obtained in this study. A third preparation, which was used to prepare fragments I and II, was obtained from pooled rabbit serum by ammonium sulfate fractionation (Kendall, 1937) followed by chromatographic purification on DEAE-cellulose (Sober and Peterson, 1958). This material was of a comparable homogeneity to that prepared by sodium sulfate fractionation, but contained 2–3% of other protein species as judged by ultracentrifugal analysis.

Specific Antibodies. Rabbit antibovine serum albumin was prepared by the method of Singer *et al.* (1960). Seventy % of the antibody was precipitable with bovine serum albumin. Rabbit antipneumococcus polysaccharide S-III antibody was prepared from hyperimmune rabbit serum kindly supplied by Miss Jessie Hendry, New York State Department of Health, Division of Laboratories and Research. It was isolated by precipitation with purified polysaccharide and dissociated from the specific precipitate with salt (Heidelberger and Kabat, 1938). Seventy-five % was precipitable with the polysaccharide. Fragment III was prepared from this antibody by hydrolysis of the antigen-antibody precipitate with papain (Gitlin and Merler, 1961).

Preparation of Fragments and Chains from γ-Globulin. Papain fragments I and III were prepared by a slight modification of the method of Porter (1959). Mercaptoethanol was employed instead of cysteine and the reaction was terminated by the addition of a 5-fold molar excess of iodoacetamide. In 20–30 minutes after termination of the reaction, the preparation was dialyzed against a large volume of cold water. Fragment III precipitated as crystals during dialysis. These crystals were recrystallized twice by dissolving at *p*H 4.5 with acetic acid and adjusting the *p*H to 7 with 0.01 M NaOH. Recrystallization did not adequately remove undigested or partially digested γ-globulin when these were present, so that fragment III had to be further purified by gel filtration. For this purpose, 6.5- × 130-cm columns of Sephadex G-200 (Pharmacia, Inc., Uppsala, Sweden) equilibrated with 0.1 M Tris-chloride buffer, *p*H 8.0, were employed. Impure fragment III (0.4–0.6 g in 25–50 ml buffer) was applied to the column and 30-ml fractions were collected at a flow rate of 30–50 ml/hr. A typical elution pattern is shown in Figure 1. Fragment I and additional amounts of fragment III were isolated from the exhaustively dialyzed mixture by chromatography on CM-cellulose according to the methods of Porter (1959). Fragment I, obtained in this manner, was not purified further. The fragments I and III prepared by these methods sedimented as homogeneous peaks on analytical ultracentrifugation and were judged to contain less than 5% impurities when electrophoresed on starch gel at *p*H 4.5 and on tryptic peptide analysis as described in the following paragraphs.

The A and B chains were prepared by the method of Fleischman *et al.* (1962) under conditions which employed the highest mercaptoethanol concentrations (0.75 M) recommended by these workers. Analysis of the purified chains on Sephadex G-200 by the method of Small *et al.* (1963) revealed between 5 and 10% contamination of A chain with the B chain.

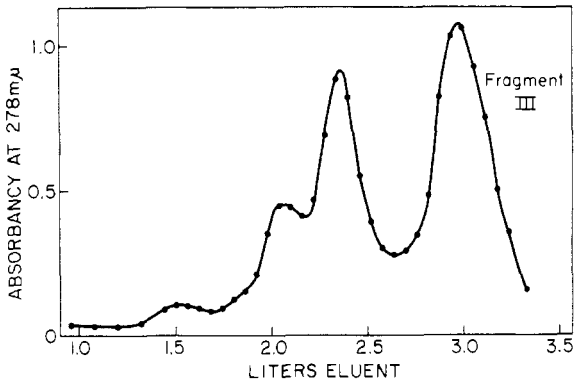

FIGURE 1: Purification of fragment III from nonspecific γ-globulin on Sephadex G-200. The fragment III present in the fraction from 2.8 to 3.3 liters was collected by lyophilization after exhaustive dialysis against water. Those peaks emerging between 1.5 and 2.5 liters represent γ-globulin and an intermediate fragment produced during digestion with papain (Nelson, 1964).

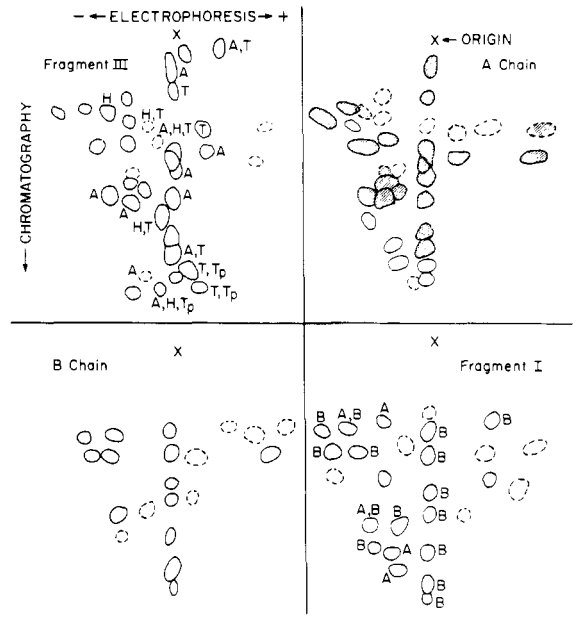

FIGURE 2: Tracings of the tryptic peptide maps of *S*-carboxymethylated chains and fragments of γ-globulin by the two-dimensional electrophoretic-chromatographic technique on paper. Those peptides which were present in all patterns are traced with a smooth line. Peptides which were observed only occasionally are indicated by the dashed line. Those peptides in the maps of fragment III which contained specific amino acids are indicated by capital letters: A = arginine, H = histidine, T = tyrosine and Tp = tryptophan. The peptides in the maps of A chain which appear to be present also in maps of fragment III are shaded. The peptides in fragment I which are derived from B chains are indicated by the letter B, and those from the A chain, by A. Other, even fainter areas were observed with greater sample loads in fragment I, B-chain, and A-chain maps.

Preparation of S-Carboxymethyl and Carboxamidomethyl Fragments and Chains. Prior to digestion with trypsin and analysis of tryptic peptides, the fragments and chains were reduced and treated with iodoacetic acid or iodoacetamide. The chains or fragments were dissolved in a solution which was 6.7 M in guanidine hydrochloride, 0.1 M in mercaptoethanol, and 0.2 M in Tris-chloride buffer, *p*H 8.0, to give a protein concentration of 20 mg/ml. The mixture was allowed to stand 12 hours at room temperature. Although it was unnecessary to perform the reduction anaerobically, the mixture was not vigorously stirred or treated in a manner which would introduce oxygen. The reduction was terminated by addition of a 2.5-fold molar[1] excess of iodoacetamide or iodoacetic acid in 6.7 M guanidine at *p*H 8. After 20–30 minutes the mixture was diluted 3-fold with water and dialyzed exhaustively against cold distilled water. Loss of protein during dialysis was minimal if acetylated dialysis tubing (Meyer and Schellman, 1962) was employed. The yields of derivative were 85–95%. Quantitative chromatographic analysis (Spackman *et al.*, 1958) of acid hydrolysates of these derivatives showed only the *S*-carboxymethylcysteine with no detectable quantities of the carboxymethyl derivatives of methionine, lysine, or histidine (Gundlach *et al.*, 1959).

Tryptic Hydrolysis. Solutions (1 or 2%) or suspensions of the alkylated chain or fragment preparations were digested with trypsin at 40° at *p*H 8–9. Trypsin (three times crystallized, trichloroacetic acid precipitated, Worthington Biochemical Corp. lot 591) in an amount equal to 1% of the weight of the protein substrate was employed in each case. The *p*H dropped rapidly during the initial stages of the digestion and was maintained between *p*H 8 and 9 by intermittent addition of 0.1 N NaOH. Although the substrates were only partially soluble at the outset of hydrolysis, all insoluble material dissolved within the first 30 minutes of hydrolysis. When the *p*H remained constant for 1 hour, hydrolysis was judged to be complete. For fragment III, hydrolysis was complete in 2–3 hours, whereas complete digestion of fragment I or the A and B chains required 8 hours. After digestion was complete, the *p*H of the mixture was adjusted to *p*H 4 with acetic acid, and the insoluble peptides which formed were removed by centrifugation. Alternatively, the digest was made 5% in dichloroacetic acid and the insoluble material was removed. The excess dichloroacetic acid was extracted from the soluble portion of the digest by extraction with ether. The soluble tryptic peptides obtained after precipitation at *p*H 4 or with dichloroacetic acid were indistinguishable from one another. If the insoluble peptides were not removed

[1] Two and one-half times the thiol content of γ-globulin plus mercaptoethanol.

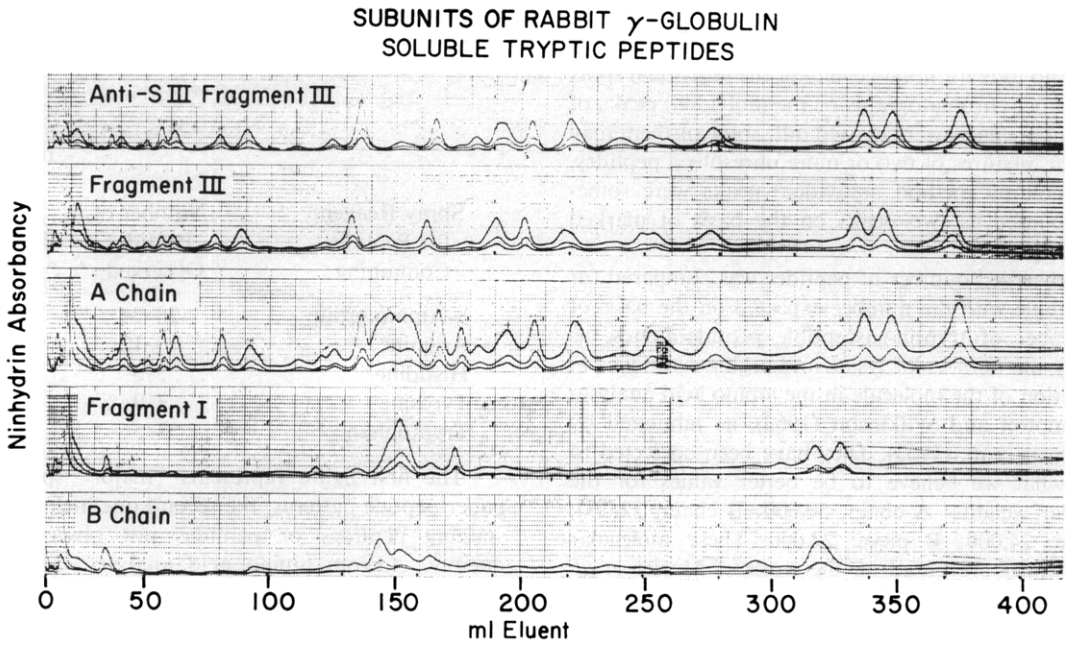

FIGURE 3: The column-chromatographic patterns of the tryptic peptides derived from *S*-carboxymethylated chains or fragments of nonspecific γ-globulin and antipneumococcus polysaccharide fragment III. Details of the gradient are described in the text.

from the tryptic hydrolysates, analysis of the tryptic peptides as described here was often of poor quality.

Analysis of Tryptic Peptides. Tryptic peptide patterns were obtained by two different methods. The two-dimensional paper electrophoretic–chromatographic method of Ingram (1958) as modified by Kimmel *et al.* (1962) was employed. Tryptic peptides corresponding to 0.25–0.35 μmole of intact chain or fragment, in a volume of 50 μl, were spotted on Whatman 3MM paper and resolved electrophoretically for 3 hours at a potential gradient of 10 v/cm in 0.2 M pyridine acetate, *p*H 6.5. The papers were dried and developed by descending chromatography for 14 hours in pyridine–1-butanol–acetic acid–water solvent (50:75:15:60). The peptides were detected with a ninhydrin spray reagent. Peptides which contained tyrosine, arginine, tryptophan, or histidine were detected with specific spray reagents described earlier (Perkoff *et al.*, 1962).

Tryptic peptides were also analyzed by the column-chromatographic method of Jones (1964). The soluble tryptic peptides were resolved at 50° on 0.9- × 15-cm columns of Dowex 50X8 ion-exchange resin at a flow rate of 30 ml/hr with a linear gradient formed with 0.2 M pyridine acetate, *p*H 3.1, as starting buffer, and 2 M pyridine acetate, *p*H 5.0, as limit buffer. The column eluates were monitored with ninhydrin in the automatic amino acid analyzer system (Spinco Model 120) of Spackman *et al.* (1958). Aliquots (1 ml) of hydrolysates containing 0.3–0.4 μmole of substrate were routinely analyzed.

Results

Analysis of Tryptic Peptides from Nonspecific γ-Globulin. Typical peptide maps of the chains and fragments of nonspecific γ-globulin are shown in Figure 2. Examination of similar tryptic digests by the column chromatographic technique of Jones (1964) is shown in Figure 3. Each result shown is only one of several independent analyses of similar material.

In the analysis of these data, an attempt has been made to distinguish between peptides obtained reproducibly in good yield and those which occur only in poor yield, or which are observed in some but not all patterns obtained with similar material. Such minor peptides are likely to arise from incomplete tryptic hydrolysis, from occasional splits at peptide bonds other than those adjacent to arginine or lysine, or from incomplete purification of the materials subjected to hydrolysis (e.g., contamination of A chain with a small amount of B chain). This distinction between important and unimportant peptides involves some subjective judgments as well as uncertainty as to the amount of variation of color yields from different peptides. The numbers given are therefore to be regarded as somewhat uncertain. This is true even for the analysis by column chromatography, where a quantitative estimate of the yield of each peptide can be made. We have counted as "major" peptides those which represent at least 1% of the total area under each trace shown in the figure. There are however always a few borderline

peaks, which may represent more than 1% in one experiment and less than 1% in another.

Since no tests for homogeneity of the individual spots of the paper chromatograms or the individual peaks of the column eluates were carried out, some of them may represent mixtures of two or more unresolved peptides. In the column method, individual peaks were sometimes counted as two peaks on the basis of marked asymmetry.

The observed number of peptides was compared for each sample with the number expected on the basis of the number of trypsin-susceptible peptide bonds, as given by the number of lysine and arginine residues in any portion of the molecule in the amino acid analyses of Crumpton and Wilkinson (1963), as tabulated by Fleischman *et al.* (1963). They have been corrected to reflect what we believe to be better values for the molecular weights: A chain, 50,000; B chain, 22,000; A piece, 24,000; B piece, 22,000. These molecular weight values are based on the work of Marler *et al.* (1964) and more recent unpublished studies by the same authors.

The best results were obtained with fragment III, all tryptic peptides of which were completely soluble at *p*H 6.5. Column chromatography of the digest showed about thirty to thirty-two peaks, twenty-four of which represented more than 1% of the integrated area under all the peaks. The paper peptide maps showed twenty-eight well-resolved spots which were present in all fragment III digests (indicated by smooth lines in the figure), and six additional spots (indicated by dashed lines), which were observed only in some patterns.

Since fragment III contains approximately fifty residues of lysine and arginine per mole, it is evident that only about half the expected number of peptides was observed. This is the expected result if fragment III consists of two polypeptide chains with identical or nearly identical sequence, and the data thus confirm the prediction of the four-chain model. Further supporting evidence was obtained by spraying the tryptic peptides on the paper patterns with reagents which specifically detect peptides containing arginine, histidine, tyrosine, and tryptophan. A summary of the number of peptides which contain these specific amino acids is given in Table I. Again, the number of unique tryptic peptides containing each of these amino acids is about half the expected number.

Analysis of the tryptic peptides of fragment I and A and B chains was complicated by the fact that a significant fraction of the tryptic digests of these materials was not soluble at *p*H 6.5. The insoluble material was removed from the digest by precipitation with dichloroacetic acid prior to separation. It will be assumed, for the purpose of discussing the results obtained with the soluble peptides, that this insoluble material does not contribute an appreciable number of distinct peptides to the total number of such peptides, i.e., it will be assumed that the number of highly insoluble peptides is very small (partly because such peptides are likely to be high in molecular weight), and that some of the precipitated material consists of peptides of moderate solubility, partial yields of which will appear in the soluble portion of the hydrolysate.

TABLE I: Summary of the Nature of Tryptic Peptides in S-Carboxymethyl Fragment III.

Spray Reagent for Peptides Containing:	Number of Peptides Observed[a]	Content of Specific Amino Acids in Fragment III[b]
α-Amino group	28–34	50
Arginine	10–12	20
Histidine	3–5	9
Tyrosine	7–9	17
Tryptophan	3–5	8

[a] The first figure represents "major" spots in the paper peptide patterns, the second one includes weakly staining peptides or peptides not observed reproducibly. [b] From amino acid analyses. See text for source of data.

The total number of distinct peptides obtained from A chain is close to the number expected on the basis of its content of thirty-nine lysine-plus-arginine residues, but only if minor as well as major peptides are counted. The actual count by column chromatography yielded forty distinct peaks, of which thirty were major peaks. The paper peptide maps showed thirty-four spots which appeared reproducibly. In addition, the paper patterns always contained faintly colored areas, the intensity of which increased when increasing amounts of sample were applied. It is possible that these areas represent poorly resolved mixtures of peptides in low yield, i.e., they are a possible indication of microheterogeneity. Faintly colored areas of this kind were never observed with fragment III. Because not all of the tryptic digest was sufficiently soluble to appear in the final solution subjected to analysis, minor peptides cannot in this instance necessarily be dismissed as unimportant. They could represent important peptides of low solubility. However, at least some of these peptides should correspond to minor peptides observed for fragment III, so that probably not all of A chain is accounted for by the peptides actually observed.

Twenty-three of the thirty major peaks observed for A chain by the column method occur at positions at which major peaks are also observed for piece III. All but one of the major peaks found for piece III are in fact accounted for in this way. Examination of the peptide maps of Figure 2 leads to a similar conclusion. Twenty-five of the thirty-four A-chain spots have corresponding spots in the fragment III pattern. About six of the twenty-eight consistent spots observed in fragment III are not found in the A-chain hydrolysate, but four of these occur in the region of the pattern which is furthest removed from the point of application of the

tryptic digest. We were not able to obtain good resolution of this region of the map in the experiments with A chain. (Note that only thirty-four spots are shown in Figure 2 in the A-chain pattern, although forty peaks are obtained by the column technique.) Both results suggest the correctness of the four-chain model, according to which fragment III is derived entirely from the A chains of γ-globulin. One or a small number of peptides unique to fragment III is expected from the region of the A chain in which the papain split occurs.

Column chromatography of the B-chain digest yielded twenty-six peaks, of which seventeen correspond to more than 1% of the total area under all peaks. The paper method yielded thirteen spots which give a good color with ninhydrin, seven weaker spots, and, in addition, faintly colored areas similar to those observed with A chain. Since B chains contain only eleven or twelve lysine and arginine residues, this result clearly indicates that the B chains of γ-globulin are heterogeneous, supporting the finding of Cohen and Porter (1964) that B chains from γ-globulins of several species are mixtures of several distinct chains, separable by electrophoresis.

Fragment I yielded nineteen major peaks by the column method and, like B chain, showed a large number (thirteen) of minor peaks. On paper there were twenty good spots, seven weaker ones, and again some faintly colored areas. Of the nineteen major peaks shown in Figure 3, six occurred at positions corresponding to six of the seven major A-chain peaks which could not be assigned to equivalent peaks from fragment III. All major A-chain peaks except one (the marked shoulder emerging at 143 ml in Figure 3) thus correspond to peaks also observed for either fragment III or fragment I, in agreement with the prediction of the four-chain model. A similar correspondence is observed in the paper peptide maps, as shown in Figure 2. Five major spots in the fragment I pattern correspond to A-chain spots. All but two of the unshaded major spots in the A-chain pattern have corresponding spots in the fragment I pattern. The two spots unique to A chain, and the single peak unique to A chain which is observed by the column method, presumably arise from that region of the A chain which is split by papain in the formation of fragments I and III.

Most of the major peaks obtained from fragment I by the column method, which do not correspond to A-chain peaks, do occur at positions close to those at which B-chain peaks occur, although there are exceptions (at 118 and 304 ml). A similar conclusion is reached from the paper maps. As Figure 2 shows, fifteen spots obtained from fragment I appear to coincide with spots observed for B chain.

Analysis of Tryptic Peptides from Two Specific Antibodies. The paper and column methods have also been employed to examine the tryptic digests from the A and B chains and fragment III from rabbit antibovine serum albumin and rabbit antipneumococcus type III polysaccharide. They are compared with corresponding patterns from nonspecific γ-globulin in Figures 3–6.

Figure 4 shows a photograph of the tryptic peptide

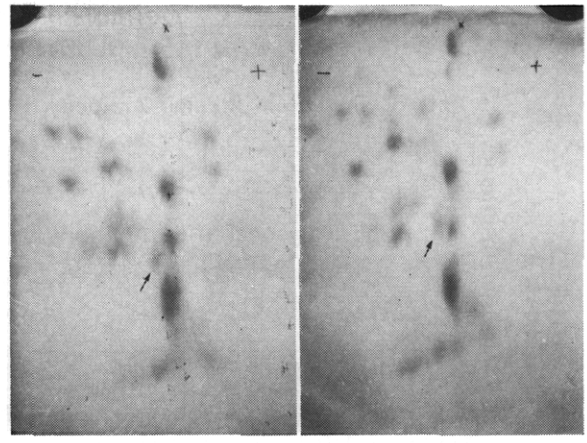

FIGURE 4: Peptide maps of tryptic digests of *S*-carboxymethylated fragment III (left) from rabbit antibovine serum albumin and rabbit antipneumococcus type III polysaccharide (right).

patterns on paper of fragment III from the two specific antibodies. Clearly all but one of the peptides are indistinguishable in the two patterns. One peptide, indicated with an arrow, having an R_F of about 0.5, migrates slightly away from the midline toward the anode in the polysaccharide antibody digests. An identical peptide is not observed for fragment III from the antibody to serum albumin, nor is it seen in the digest from nonspecific γ-globulin in Figure 2, although a corresponding peptide which is in the midline with an R_F of about 0.6 is observed. It is possible that this difference reflects genetic differences in the rabbits used in these studies. (The antibody to the polysaccharide was prepared more than 25 years ago at the New York Department of Health.) A similar conclusion is reached from the column chromatograms shown in Figure 3. There is only one significant difference between the patterns for fragment III from the antibody and that from nonspecific γ-globulin, and this lies in the part of the mixture which emerges between 190 and 220 ml. A peptide emerging just above 190 ml in the antibody digest is absent in the pattern for nonspecific fragment III.

The column-chromatographic patterns of tryptic digests of the A chains and B chains from the two antibodies and nonspecific γ-globulin are shown in Figures 5 and 6. The A-chain patterns from the specific antibodies differ slightly from one another and from the pattern given by the tryptic digest of A chains of nonspecific γ-globulin. This is most apparent for peaks at 94, 123, 206, and 261 ml of gradient. Because the height and shape of these peaks varied from one run to another, it is not certain that major structural differences are reflected by these differences. Some acidic peptides, such as those at 39–41 ml and in the 50- to 60-ml fraction, differ somewhat and were quite reproducible. Most of these differences presumably arise from that portion of the A chain which is not a part of fragment III. However, a direct comparison with the peptides

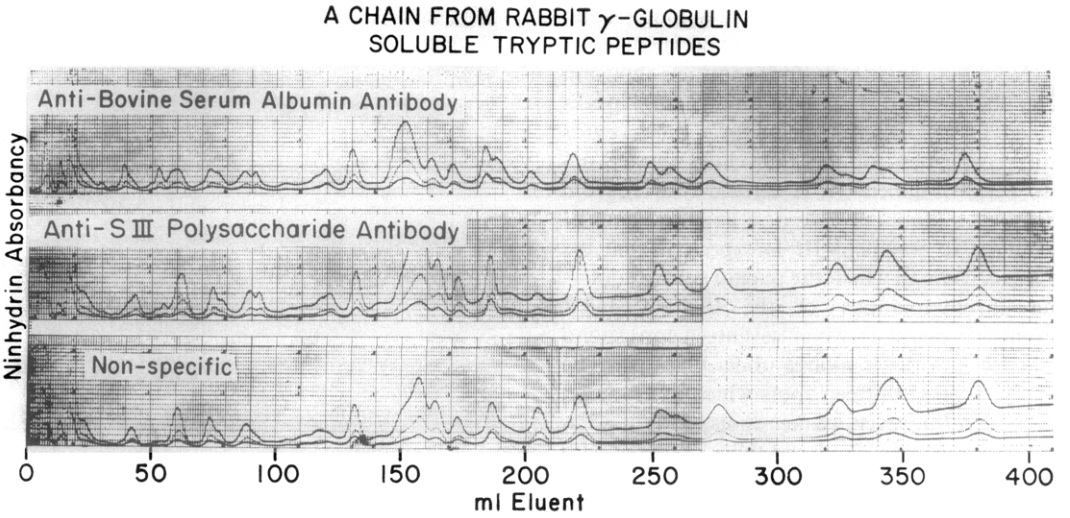

FIGURE 5: Column chromatographic patterns of the *S*-carboxamidomethylated A chains from nonspecific γ-globulin, antibovine serum albumin, and antipneumococcus type III polysaccharide. Details of the analyses are described in the text.

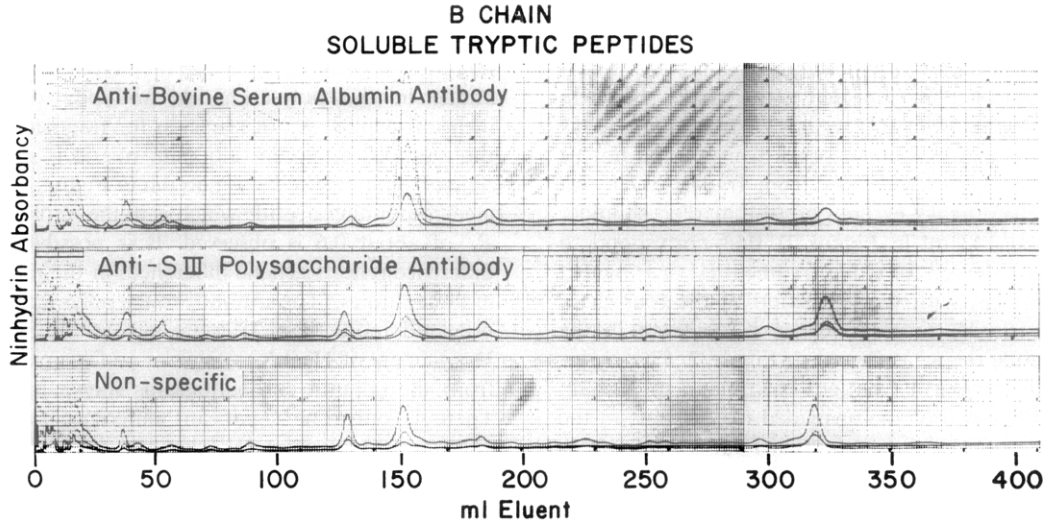

FIGURE 6: Column chromatographic patterns of the *S*-carboxamidomethylated B chains from nonspecific γ-globulin, antibovine serum albumin, and antipneumococcus type III polysaccharide. Details of the analyses are described in the text.

from fragment III is not possible, since the data given in Figures 5 and 6 were obtained with a carboxamidomethylated rather than a carboxymethylated sample.

The patterns shown in Figure 6 for B-chain digests are remarkable in that they show such striking similarities in the major peaks. Clearly the microheterogeneity of B chains, discussed earlier, is not primarily a result of major extensive differences in the structure of B chains from different antibodies. Because so many of the peptides obtained from B chains lie near the borderline (1% of total yield) which we have used as a criterion for real structural significance, a discussion of the differences which Figure 6 does show does not seem appropriate at this time.

Discussion

The results obtained in this study with chains and fragments of nonspecific γ-globulin support the four-chain structure (Porter, 1962) now generally accepted for this protein (Edelman and Gally, 1964). We have found that most peptides from fragment III correspond to similar peptides obtained from A chain. Furthermore, the number of peptides obtained from fragment

III indicates that it consists of two polypeptide chains which are identical, or nearly so, in their amino acid sequence. Similarly, most peptides from fragment I correspond either to B-chain peptides or to those A-chain peptides which do not have corresponding fragment III peptides. Peptides which occur in significant yield uniquely in the digest of fragments and do not correspond to peptides from any chain (and vice versa) are small in number and can generally be assumed to arise from that portion of the A chain which is split by papain in the preparation of the fragments. All of these results are in good agreement with published data obtained by similar methods in other laboratories (Seijen and Gruber, 1963; Givol and Sela, 1964; Haurowitz and Gross, 1964).

When the peptide maps of the chains and fragment III from two specific antibodies are compared to maps of similar materials from nonspecific γ-globulin, it is striking that only small differences are observed. As far as fragment III is concerned, this result is not surprising since this part of the antibody molecule does not contain the biologically active site. Lack of specificity in the peptide patterns derived from fragment III has been reported previously (Givol and Sela, 1964; Haurowitz and Gross, 1964).

On the other hand, some differences might have been expected in the patterns of A and B chains, since they contain the antibody-binding site, which implies that they contain regions specific in amino acid sequence (Haber, 1964; Whitney and Tanford, 1965). No direct evidence for such antibody-specific sequences was found, but there is possible indirect evidence in the fact that, of the thirty major peaks found by column chromatography of A-chain peptides from nonspecific γ-globulin, twenty-three correspond to fragment III peptides and only six to major fragment I peptides, and the similar numbers derived from the paper patterns: thirty-four spots in all, twenty-six corresponding to fragment III spots and five to fragment I spots. By amino acid analysis there are thirty-nine lysine-plus-arginine residues in A chain, of which about twenty-five are in that part of the A chain which is part of fragment III, and fourteen in that portion which is part of fragment I. Evidently sufficient peptides were not recovered from A chain to account for the whole structure, and the missing peptides all come from the fragment I part of the molecule, which contains the active site. This could mean that there are many different sequences in this part of the molecule, none of which is present in appreciable amount. Differences between antibodies of different specificity could lie in these intrinsically hard-to-detect peptides. However, such an interpretation of the A chain pattern is purely speculative. The missing peptides of the A chain of nonspecific γ-globulin could equally well be in the insoluble part of the tryptic digest, which was removed before analysis. This digest could contain enough peptides to account for the entire structure of nonspecific A chain in terms of unique peptides.

It should be noted in this connection that Givol and Sela (1964) found peptide maps of pepsin- and Nagarse-hydrolyzed fragments I and II from nonspecific γ-globulin and from two antibodies to be very similar, though some differences were observed. The peptide mixtures studied by them were entirely soluble, but, because of the lack of specificity of the proteolytic enzymes which they used, their data cannot indicate whether the patterns obtained can account for all or only a part of the structure of the fragments.

Possible support for the speculation that some portion of the A chain in fact does not yield peptides in detectable amounts is afforded by a marked contrast between our results for A chain and B chain. For both chains, part of the tryptic hydrolysate is insoluble. In the case of B chain, however, the total number of peptides is in excess of the number expected on the basis of the number of trypsin-susceptible bonds. Even the number of "major" peaks is on the high side of the expected number. In the case of A chains, the number of "major" peptides alone is well below the expected number, and even the total number of all peptides observed is on the low side.

Whether or not a portion of the A chain in fact possesses an enormous number of possible amino acid sequences, such as expected on the basis of some of the theories for the generation of antibody specificity (Burnet, 1957; Lederberg, 1959), cannot be decided without much further study. On the other hand, one fact which emerges unquestionably is that much of the A chain does appear to have a unique structure common to antibodies and nonspecific γ-globulin alike. This section of the A chain comprises all or most of the portion of the A chain which forms part of fragment III, but some peptides from fragment I must also be included in this category. The differences in sequence which may exist to confer specificity upon the A chain thus appear to arise from a relatively small portion of that chain.

Regarding the B chain, our results offer support for microheterogeneity of the type suggested by the gel-electrophoresis studies of Edelman *et al.* (1963) and Cohen and Porter (1964). These studies indicate that there is a small number of distinct B chains (about ten), at least some of which are present in roughly equal quantities in both nonspecific γ-globulin and in specific antibodies. No information can be obtained from our data on the question of whether a portion of the B chain might in addition have a widely varying sequence, associated with antibody specificity, and thus lead to no detectable peptides at all. This and other questions raised in this paper are the subject of continuing investigation.

References

Buckley, C. E., III, Whitney, P. L., and Tanford, C. (1963), *Proc. Natl. Acad. Sci. U.S. 50*, 827.

Burnet, F. M. (1957), *Australian J. Sci. 20*, 67.

Cohen, S., and Porter, R. R. (1964), *Biochem. J. 90*, 278.

Crumpton, M. J., and Wilkinson, J. M. (1963), *Biochem. J. 88*, 228.

Day, L. A., Sturtevant, J. M., and Singer, S. J. (1963), *Ann. N.Y. Acad. Sci. 103*, 611.
Edelman, G. M., Benacerraf, B., and Ovary, Z. (1963), *J. Exptl. Med. 118*, 229.
Edelman, G. M., and Gally, J. A. (1964), *Proc. Natl. Acad. Sci. U.S. 51*, 846.
Eisen, N. H., and Siskind, G. W. (1964), *Biochemistry 3*, 996.
Feinstein, A., Gell, P. G. H., and Kelus, A. S. (1963), *Nature 200*, 653.
Fleischman, J. B., Pain, R. H., and Porter, R. R. (1962), *Arch. Biochem. Biophys.* (Suppl. 1), 174.
Fleischman, J. B., Porter, R. R., and Press, E. M. (1963), *Biochem. J. 88*, 220.
Gitlin, D., and Merler, E. (1961), *J. Exptl. Med. 114*, 217.
Givol, D., and Sela, M. (1964), *Biochemistry 3*, 451.
Gundlach, H. G., Stein, W. H., and Moore, S. (1959), *J. Biol. Chem. 234*, 1754, 1761.
Haber, E. (1964), *Proc. Natl. Acad. Sci. U.S. 52*, 1099.
Haurowitz, F., and Gross, F. L. (1964), Abstracts of Papers, 143rd Meeting of the American Chemical Society, Cincinnati, Ohio, 1964, p. 12A.
Heidelberger, M., and Kabat, E. M. (1938), *J. Exptl. Med. 67*, 181.
Ingram, V. M. (1958), *Biochim. Biophys. Acta 28*, 539.
Jones, R. L. (1964), *Cold Spring Harbor Symp. Quant. Biol. 29*, 297.
Kekwick, R. A. (1940), *Biochem. J. 34*, 1248.
Kendall, F. E. (1937), *J. Clin. Invest. 16*, 921.
Kimmel, J. R., Kato, G. K., Paiva, A. C. M., and Smith, E. L. (1962), *J. Biol. Chem. 237*, 2525.
Koshland, M. E., and Englberger, F. M. (1963), *Proc. Natl. Acad. Sci. U.S. 50*, 61.
Lederberg, J. (1959), *Science 129*, 1649.
Marler, E., Nelson, C. A., and Tanford, C. (1964), *Biochemistry 3*, 279.
Meyer, Y. P., and Schellman, J. A. (1962), *Biochim. Biophys. Acta 55*, 361.
Nelson, C. A. (1964), *J. Biol. Chem. 239*, 3727.
Nelson, C. A., Noelken, M. E., Buckley, C. E., III, Tanford, C., and Hill, R. L. (1963), *Federation Proc. 22*, 657.
Palmer, J. L., Mandy, W. J., and Nisonoff, A. (1962), *Proc. Natl. Acad. Sci. U.S. 48*, 49.
Pauling, L. (1940), *J. Am. Chem. Soc. 62*, 2643.
Perkoff, G. T., Hill, R. L., Brown, D. M., and Tyler, F. H. (1962), *J. Biol. Chem. 237*, 2820.
Porter, R. R. (1959), *Biochem. J. 73*, 119.
Porter, R. R. (1962), *Basic Probl. Neoplastic Disease, Symp. New York, 1962*, 177.
Seijen, H. G., and Gruber, M. (1963), *J. Mol. Biol. 7*, 209.
Singer, S. J., Fothergill, J. E., and Shainoff, J. R. (1960), *J. Am. Chem. Soc. 82*, 565.
Small, P. A., Jr., Kehn, J. E., and Lamm, M. E. (1963), *Science 142*, 393.
Smithies, O. (1959), *Biochem. J. 71*, 585.
Sober, H. A., and Peterson, E. A. (1958), *Federation Proc. 44*, 1116.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Whitney, P. L., and Tanford, C. (1965), *Proc. Natl. Acad. Sci. U.S. 53*, 524.