A COMPARISON OF THE PEPTIDES RELEASED FROM RELATED RABBIT ANTIBODIES BY ENZYMATIC HYDROLYSIS*, ‡

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Current theories of antibody formation may be resolved, for the most part, into three different concepts: (*a*) An antibody is formed by a specific modification of the tertiary structure of certain globulins, the folding of the globulin molecules being influenced by the antigen (2–4); in this case, the primary structure of the altered globulin is the same as that formed in the absence of antigen and the specificity of the antibody molecule resides in the formation of areas during folding that are complementary to the antigen (4, 5). (*b*) Antibodies arise through modifications of the genetic material responsible for protein specificity; in this concept, the genetic alteration is induced by antigen and results in the synthesis of globulins specifically reactive with the antigen (6, 7). (*c*) Antibodies appear through selective proliferation, induced by antigen, of cells genetically preinvested with the synthesis of proteins complementary to the antigen (8, 9); according to this view, a large number of different γ-globulins (8) or cell "receptors" (9) are normally synthesized and those cells which produce γ-globulins or cell "receptors" that can react with a given antigen proliferate after contact with that antigen.

Since some of the genetically induced protein modifications thus far known have been related to alterations in amino acid sequence or primary structure (10–12), it has been presumed that if antibody formation is genetically determined or is the result of a genetic mutation, different specific antibodies would have different primary structures (12 *a*). Thus, differing concepts of antibody structure have become associated with different theories of antibody synthesis. It seemed of interest, therefore, to compare the primary structures of related purified antibodies by studying the peptides released from them during enzymatic hydrolysis.

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Materials and Methods

Rabbit Anti-Pneumococcus Antibodies.—The antisera used in this study were kindly supplied by Dr. H. D. Piersma of Lederle Laboratories and had been prepared by immunization of rabbits with formalin-killed pneumococci. Each lot of antiserum was specific for a single pneumococcus type and each lot contained the pooled sera of 2000 to 6000 rabbits. The pooled rabbit sera had been repeatedly fractionated with one-third saturated ammonium sulfate to obtain a highly purified and concentrated antibody fraction. Approximately 95 per cent of the protein in the resulting antisera was γ-globulin by electrophoresis and at least 95 per cent of the protein was precipitable with a bovine anti-rabbit γ-globulin antiserum. From 15 to 30 per cent of the protein in these antisera was precipitable with the homologous pneumococcus capsular polysaccharide.

Antibodies against pneumococcus polysaccharides of Types II, III, VI, VII and XII were isolated from the type-specific antisera by precipitation with homologous antigen in the equivalence zone; the pneumococcus polysaccharides used as antigens had been prepared by the method of Heidelberger, Kendall, and Scherp (13). After incubation of antigen and antiserum at 37°C for 1 hour and then at 2°C for an additional 24 hours, the antigen-antibody precipitates were collected by centrifugation at 3000 RPM for 30 minutes at 0°C and carefully washed four times in 0.15 M NaCl. Antibodies against Types II, III, and XII polysaccharides were obtained free of antigen from aliquots of the antigen-antibody precipitates by the method of Heidelberger and Kendall (14). These antigen-free antibodies appeared to be homogeneous in the ultracentrifuge and had a sedimentation constant, $S_{w,20}$ of 6.6S.

At least two separate isolations of a specific antibody from a given lot of antiserum were performed. Antibodies against Types II, VII and XII polysaccharides were each isolated from *two* different lots of homologous antiserum, but each of the others were isolated from single lots of homologous antiserum.

The purified antigen-free antibodies, the specific precipitates and the γ-globulin remaining in the antisera after removal of the specific precipitates were all dialyzed against several changes of distilled water for 2 days.

Enzymatic Hydrolysis.—The subtilisin used in this study was prepared from a strain of *B. subtilis* by the Novo Company in Copenhagen and was kindly supplied by Dr. M. Ottesen of the Carlsberg Laboratory. The chymotrypsin, trypsin, and papain used were obtained from the Worthington Biochemical Company, Freehold, New Jersey, and each had been crystallized twice. All enzymatic hydrolyses were performed in the pH stat at 37°C, the pH being kept constant during hydrolysis by the addition of NaOH (15). When subtilisin, chymotrypsin, and trypsin were employed, hydrolysis was performed at pH 8.0 and the enzyme to protein ratio was 1:30 by weight. For papain hydrolysis, pH was maintained at 7.0, the time for hydrolysis was kept between 10 and 20 minutes and the enzyme to protein ratio was 1:1000 by weight. Digestions of each antibody or γ-globulin preparation were performed at least in duplicate.

The following hydrolysates were prepared: (*a*) Aliquots of each of the dialyzed antigen-free antibodies, specific precipitates and γ-globulins remaining after removal of the specific precipitates were adjusted to pH 8.0 with NaOH, heated to 100°C for 15 to 20 minutes in a water bath and then digested with subtilisin. (*b*) Aliquots of Types II, VI, and VII specific precipitates were hydrolyzed with subtilisin immediately after dialysis without prior denaturation. (*c*) Aliquots of Types VI, VII, and XII specific precipitates were oxidized with performic acid (16) and then hydrolyzed with either subtilisin, chymotrypsin or trypsin. (*d*) Aliquots of dialyzed Types VI, VII, and XII specific precipitates, and the γ-globulin remaining after removal of the specific precipitates were adjusted to 0.1 M KCl, 0.01 M cysteine, and 0.002 M ethylenediaminetetraacetate (17) and hydrolyzed with papain. The papain hydrolysates were dialyzed

against several changes of distilled water at 2°C over a period of 1 to 2 days and the dialyzable materials, fraction D, were lyophilized. Crystals formed in the non-dialyzable, portion of the hydrolysates as described by Porter (17); these were collected, dissolved in water, and recrystallized twice (fraction III). The remainder of the non-dialyzable material was fractionated using a carboxymethyl cellulose column, sodium acetate-acetic acid buffer pH 5.5 in a concentration gradient from 0.005 to 0.9 M, and fractions I and II were collected (17). (*e*) Aliquots of fractions I, II and III were oxidized with performic acid (16) and hydrolyzed with either subtilisin or chymotrypsin.

After hydrolysis with subtilisin, chymotrypsin, or trypsin, the hydrolysates were adjusted to pH 4.2 with 0.7 N HCl, lyophilized, and then redissolved in water to a concentration of 50 to 100 mg of original protein per ml.

Separation of Peptides.—Separation of the peptides present in the hydrolysates was performed as described elsewhere (18). Briefly, 2 to 4 μl of hydrolysate, equivalent to 0.1 to 0.4 mg of peptides, were applied to an area 1 by 7 mm on a sheet of Whatman No. 1 filter paper measuring approximately 23 x 57 cm. The paper was wet and washed with pyridine–acetic acid buffer, pH 4.2 (0.5 per cent pyridine in 0.2 M acetic acid). Separation of peptides in one dimension was accomplished by electrophoresis under cooled varsol No. 1 (Esso Standard Oil Company) using 52 volts per cm along the long axis of the paper for 45 to 90 minutes. The temperature of the Varsol never exceeded 32°C and was usually between 23 and 28°C at the end of each run. The peptides were separated in the second dimension by ascending chromatography in 2,4,6-collidine, 2,6-lutidine, and water in a ratio of 1:1:1 by volume.

Detection of Peptides.—The dried papers were sprayed with ninhydrin (0.25 per cent in ethanol) and the color developed by heating to 70°C for 15 to 30 minutes followed by room temperature for 1 to 3 days. Color reactions for the detection of tryptophane (19), tyrosine (20), or histidine (21) were also used. An approximation of the relative peptide bond concentrations was obtained in some instances by a modification of the procedure of Rydon and Smith (22): after exposure to Cl_2, the chromatograms were washed in 95 per cent ethanol, dried at 50°C and allowed to hang at room temperature for 15 minutes; the papers were then dipped into a fresh solution of KI in starch. All peptide patterns were photographed by reflected light using a light green lens filter and high contrast fine grain film.

Substances derived from the pneumococcus polysaccharides were not detected in the chromatograms under the conditions of study used here.

Hydrolysis of Selected Peptides and Amino Acid Separation.—When peptides were isolated from the two dimensional chromatograms, the chromatograms were prepared as described above but using Whatman 3 MM paper and 0.5 to 1 mg of peptides; electrophoresis was at 26 volts per cm for 60 to 100 minutes. The peptides were located with a spray of 0.025 per cent ninhydrin in ethanol.

Groups of peptides were also isolated from unidimensional separations of the hydrolysates. From 0.5 to 1 mg of peptides was applied to an area 8 cm long and 1 to 2 mm wide on 3 MM paper and electrophoresis was performed at 26 volts per cm for 60 to 120 minutes. Two hydrolysates being compared were placed side by side on the same paper. After electrophoresis, a strip 1 cm wide was cut from each end of the area of application for the entire length of the paper and sprayed with 0.25 per cent ninhydrin; the peptides were then located in the untreated 6 cm of the electrophoretograms.

The selected peptides or groups of peptides were cut from the paper, along with analogous areas from the patterns being compared, were then eluted from the paper with 0.1 M acetic acid and hydrolyzed with 0.5 ml of 5.9 N HCl in a sealed tube for 18 hours at 105°C. The solution was diluted to 5 ml, lyophilized and the amino acids redissolved in water and again lyophilized. The amino acids were separated on No. 1 filter paper using either water-saturated phenol in an ammonia atmosphere or *N*-butanol–acetic acid–water (4:1:5 by volume) in

the first dimension and collidine-lutidine-water (1:1:1 by volume) in the second dimension. The chromatograms were sprayed with 0.25 per cent ninhydrin in ethanol and the colors developed at 70°C for 20 minutes.

RESULTS

Hydrolysis with Subtilisin.—Approximately 100 to 110 peptides were differentiated in the subtilisin hydrolysates of antibodies against Types II, III, VI, VII, and XII polysaccharides when the antibodies were heat-denatured or performic acid oxidized prior to hydrolysis (Figs. 3 to 6). Assuming the molecular weight of these antibodies to be 165,000 (23), an average of about 210 μmoles of NaOH per μmole of antibody were needed to maintain pH at 8.0 during hydrolysis; the range was 198 to 245 μmoles of NaOH per μmole of antibody. Thus, only one-half of the number of peptides that were expected from the amount of base utilized during hydrolysis (24) could be distinguished in the peptide patterns.

The peptide patterns obtained for hydrolysates of heat denatured antibodies revealed reproducible differences between these antibodies in at least three peptides (Figs. 3 to 5): all three peptides were present in anti-II and anti-III antibodies (Fig. 3), only two were found in anti-XII antibodies and only one was detected in anti-VI and VII antibodies (Figs. 4 and 5). Since the peptide patterns obtained with specific precipitates of anti-II, anti-III, and anti-XII antibodies were the same as those obtained for the homologous antigen-free antibodies, the peptide differences did not appear to be attributable to the presence of antigen during denaturation. That these differences might have been due to slight differences in the degree of denaturation of the different antibodies was indicated by the observation that the peptide patterns for anti-VI, anti-VII, and anti-XII antibodies that were *not* denatured prior to hydrolysis contained two or all three peptides (Figs. 1 and 2). In the latter peptide patterns, however, other peptide differences between different antibodies were apparent (Figs. 1 and 2). When the antibodies were oxidized with performic acid before hydrolysis (Fig. 6), definite reproducible differences between the peptide patterns could not be discerned.

Although rabbit γ-globulin contains approximately 20 tryptophane residues per molecule (25), only 5 peptides containing tryptophane were distinguished in the peptide patterns for unheated antibodies and only 4 in those for heat denatured antibodies; of approximately 54 tyrosine residues, only 7 peptides containing tyrosine were found in the patterns for unheated antibodies and only 6 in those for heat denatured antibodies. Twelve of the 15 histidine residues in rabbit γ-globulin were found in different peptides. No differences were noted among different antibodies in the relative positions of these peptides; as was expected, their relative positions in the patterns did vary with differences in the extent of denaturation of the antibodies before hydrolysis.

Hydrolysis with Chymotrypsin.—Approximately 60 to 70 peptides were resolved in chymotrypsin hydrolysates of performic acid oxidized Types VI, VII, or XII antibodies (Figs. 7 to 9): on the average, about 150 μmoles of NaOH per μmole of antibody were used to maintain pH constant during hydrolysis with chymotrypsin. Thus, only about one-half the number of peptides were differentiated in the peptide patterns of these hydrolysates than was expected either on the basis of the amount of NaOH utilized or on the basis of a total of approximately 123 tyrosine, phenylalanine, and tryptophane residues per molecule of rabbit antipneumococcal antibody (25).

Definite reproducible differences between the peptide patterns for these antibodies could not be found.

Hydrolysis with Trypsin.—Approximately 50 peptides could be distinguished in trypsin hydrolysates of performic acid oxidized Types VI or VII antibodies (Figs. 11 and 12). An average of 110 μmoles of NaOH per μmole of antibody were utilized during hydrolysis to maintain pH at 8.0. As with chymotrypsin and with subtilisin, the number of peptides differentiated in the patterns was only about one-half that expected on the basis of the amount of NaOH used. The number of peptides observed was but one-half of the number to be expected on the basis of 100 to 110 moles of arginine plus lysine per mole of antibody (25).

When the trypsin hydrolysates were adjusted to pH 4.2 after digestion, about 15 per cent of the weight of the original antibody precipitated (R_1): this was unlike the hydrolysates obtained with subtilisin or chymotrypsin in which little or no precipitate formed. After 24 hours at 2°C, an additional 5 per cent of the original antibody precipitated (R_2). Of the 80 per cent that remained soluble at pH 4.2, no reproducible differences could be found between the patterns for Type VI antibody and those for Type VII antibody (Figs. 11 and 12). The portion that precipitated at pH 4.2 was soluble at pH 8.0; electrophoresis of this fraction in sodium barbiturate buffer at pH 8.0 revealed at least 7 peptides, all of which gave little or no color with ninhydrin and very intense color with Cl_2 followed by KI in starch (Fig. 10). No component resembling the original protein electrophoretically or immunochemically could be found.

Hydrolysis with Papain.—During hydrolysis of the antibodies with papain at pH 7.0, an average of 2.9 μmoles of NaOH were required to maintain constant pH. Since most of the α-amino groups released by hydrolysis become ionized at this pH and thus bind much of the hydrogen ions released during hydrolysis, the amount of base utilized suggested hydrolysis of many more than simply 3 peptide bonds. In accord with this, the dialyzable fraction of the hydrolysates which comprised 10 to 15 per cent of the weight of the antibodies yielded peptide patterns in which approximately 35 peptides were resolved (Fig. 32). No differences in these peptide patterns could be discerned among the antibodies in different specific precipitates or between the specific precipitates and the non-specific γ-globulins of the antisera.

The non-dialyzable fraction of the papain hydrolysates prepared from the purified γ-globulin fractions of the antisera to which no antigen had been added had a sedimentation constant of 4.0S. The undigested γ-globulins had a sedimentation constant of 6.6S. Using specific precipitates as the source of the hydrolysates, two components, A and B, were observed in the ultracentrifuge (Fig. 13): one with a sedimentation constant of 4.0S and the other with a greater sedimentation constant. As the amount of antigen relative to the amount of antibody was increased in the system by simply adding homologous antigen to the hydrolysate, the sedimentation constant of component B decreased (Table I and Figs. 13 to 23), as expected for an antigen-antibody complex (26), without significantly affecting the amount or the sedimentation of component A. Fraction III, the crystallized fraction from the papain hydrolysate, behaved as did component A in the presence of homologous antigen and fraction I plus II behaved as did component B, the faster component. As observed by Porter (17), fraction III did not appear to combine with antigen and fractions I and II both had sites capable of combining with antigen.

In the ultracentrifugal patterns, the relative amounts of the two components were constant when the amount of the faster component was corrected for the amount of antigen in the system. The ratio of the weights of fraction III to fractions I and II, assuming the fractions to have the same specific refractive index increment (27), was 1:1.43. Thus, polypeptide III, as indicated by Porter (17), represented approximately 35 per cent of the antibody molecule, fragments I plus II accounted for 50 per cent of the antibody molecule and the dialyzable peptides constituted 10 to 15 per cent of the antibody molecule. Since polypeptides I and II each had sedimentation constants of 4.0 and, as will be shown later, are almost identical in peptide composition, each appeared to represent 25 per cent of the antibody molecule. A mixture of purified antibody and homologous antigen in a molar ratio of 1.63:1 (antibody 165,000 molecular

TABLE I

Sedimentation Constants of Complexes of Pneumococcus Type VII Polysaccharide with Purified Rabbit Antibodies Hydrolyzed with Papain

Antibody: Antigen	S_{w20}
*molar ratio**	
22.3	49.8
22.3	35.0
22.3	33.7
11.12	17.5
11.12	16.4
8.71	18.6
4.46	11.5
1.63	9.0

* Moles of antibody, 165,000 molecular weight; moles of polysaccharide, 150,000 molecular weight.

weight: Type VII polysaccharide, 150,000 molecular weight, Table I) after digestion with papain revealed only a single component B in the ultracentrifuge suggesting that each of the antigen-combining antibody fragments resulting from papain hydrolysis had a single combining site.

A. Hydrolysis of Fractions with Subtilisin.—The hydrolysis of performic acid oxidized fraction III with subtilisin required from 70 to 90 μmoles of NaOH per μmole of fraction III to maintain pH at 8.0; the molecular weight of fraction III was taken as 80,000 (27). Approximately 70 to 75 peptides could be resolved in the peptide patterns of these hydrolysates (Fig. 28). The hydrolysis of performic acid oxidized fraction I or II each required about 60 μmoles of NaOH per μmole of the fraction to maintain constant pH; the molecular weight of fraction I or II was taken as 55,000 (27). Approximately 70 peptides were resolved in the peptide patterns for either fraction I or II (Figs. 24 to 27).

The peptide patterns of fractions I and II, while quite different from those of fraction III, were almost identical with each other. Areas of overlapping of peptides from fractions I and II with those of fraction III were evident. It would appear that about half of the spots in the patterns obtained for *total* antibody contained peptides derived

from at least two different parts of the same molecule. In accord with this, the number of peptides released by subtilisin from the three fractions was more than 210 while only about 110 peptides were resolved in the patterns of analogous total antibody digested with subtilisin. Performic acid oxidation alone did not release peptides from the antibody fractions.

The peptide patterns of fractions I and II obtained from the same type-specific antibody were remarkable for the similarity of their peptide patterns (Figs. 25 and 26). The peptide patterns for these fractions from one antibody were different with respect to at least two peptides from those of a different antibody (Figs. 25 and 27). The peptides in question, together with analogous areas from the patterns being compared, were isolated and hydrolyzed; it was found that the peptide differences reflected differences in amino acid composition (Figs. 30, 31, 40, and 41). These differences appeared to be qualitative in that amino acids were detected in the hydrolysates of one peptide that could not be found in the hydrolysate of the analogous peptide for a different antibody, although most of the amino acids in the two peptides were the same. Interestingly, however, some differences were also noted between the crystals, or fraction III, obtained from different antibodies (Fig. 29).

B. Hydrolysis of Fractions with Chymotrypsin.—Hydrolysis of performic acid oxidized fraction III with chymotrypsin required approximately 50 to 60 μmoles of NaOH per μmole of the polypeptide to maintain pH constant. In the peptide patterns of these hydrolysates, 50 to 55 peptides could be differentiated (Fig. 39). During hydrolysis of either fraction I or II, 30 to 35 μmoles of NaOH per μmole of polypeptide were used and 35 to 40 peptides could be resolved in the peptide patterns of each hydrolysate (Figs. 34 to 37). Since, as will be shown, the peptide patterns of fractions I and II were almost identical and areas of overlapping of peptides from fractions I and II with those of III were apparent, it would appear that approximately half of the spots in the patterns obtained for *total* antibody hydrolyzed with chymotrypsin contained peptides from at least two different parts of the antibody molecule. In accord with this, approximately 60 to 70 peptides were resolved in the patterns of *total* antibody digested with chymotrypsin while more than 120 peptides were released by chymotrypsin from the three fractions.

Although fractions I and II from the same antibody were similar in their peptide patterns, they were not quite identical and some reproducible though small differences were noted (Figs. 34 to 37). The patterns for fractions I and II were quite different from those of fraction III. The peptide patterns of all three polypeptide fractions showed significant differences between different antibodies (Figs. 33 to 38). That the differences were not due to differences in the γ-globulin pools from which the antibodies were obtained was indicated by the observation that the peptide patterns for the antibodies were different from those for the γ-globulins remaining after precipitation of the antibodies.

DISCUSSION

The results obtained in this study suggest, *but do not prove*, that there may be small differences in both primary and tertiary structure between antibodies against different pneumococcus polysaccharides. In support of the view that

different antibodies may have different tertiary structures are the observations made on the hydrolysis of unheated and heat-denatured antibodies with subtilisin: in different unheated antibodies, significant differences were noted and with heat denaturation, these differences disappeared while other differences were found. No definite differences were observed between these antibodies when they were performic acid–oxidized prior to hydrolysis and in these peptide patterns, the peptides in question in unheated and heat-denatured antibodies were absent. In support of the view that different antibodies may have different primary structures are the observations on the hydrolysis of performic acid–oxidized polypeptide fragments obtained from different antibodies with papain: differences in peptide patterns associated with differences in the amino acid composition of the differing peptides were found between different antibodies. Differences were noted between different antibodies for all three fractions, the crystallizable fraction without antibody sites as well as the two fractions containing antibody sites. In considering the significance of the peptide differences observed, it should be noted that the peptide pattern for a given type-specific antibody was reproducible whether replicate isolations of the antibody were made from the same or from different antiserum pools. In addition, the peptide patterns of the antibodies were independent of the other γ-globulins in the antisera. The apparent absence of detectable significant differences between hydrolysates prepared from the whole antibody molecule and the finding of differences between analogous papain fractions of the antibodies are not contradictory; the great overlapping of peptides from different parts of the molecules in the hydrolysates of whole antibody molecules would make detection of differences difficult indeed.

On the other hand, despite the fact that the antibodies being compared were handled in the same manner at the same time, *it must be emphasized* that the possibility remains that the differences observed might be attributable either to differences in the state of denaturation of the different antibodies or to differences induced by either lack of specificity or irregularity of hydrolysis by the enzymes. In the case of the antibody fractions, the fractions had been derived from hydrolysates prepared by hydrolysis of presumably undenatured antibodies with papain; any denaturation of an antibody or longer action of papain on an antibody might result in differences between fractions from different antibodies. Certainly, the three large polypeptide fragments released from undenatured antibodies with papain were not found in papain hydrolysates of heat-denatured antibodies and a large number of smaller peptides were released instead. In addition, the action of performic acid during oxidation could conceivably induce peptide differences, despite the regularity of appearance of these differences. Therefore, while the data suggest that differences in primary and tertiary structure exist between different but related antibodies, it is also apparent that much more work remains to be done before the existence of such differences are firmly established.

It should be noted that while a given antibody isolated from one antiserum pool gave the same peptide pattern as antibodies of the same type specificity isolated from another antiserum pool, this does not mean that a given specificity is necessarily associated with a single specific peptide structure. Each antiserum pool contained antibodies from 2000 to 6000 rabbits immunized with a single type of pneumococcus. Such large pools were used in order to submerge non-antibody differences in γ-globulins, such as those responsible for allotypy (28), from individual rabbits. The same procedure, however, also averages any possible differences in the peptide structures of different antibody molecules with the same type specificity, whether the antibody molecules came from the same or from different rabbits. In addition, the rabbits used for these antiserum pools had been immunized for prolonged periods of time before being bled; this study does *not* reveal whether antibodies appearing early in the course of antigenic stimulation might have different peptide structures from antibodies appearing later.

The differences in primary and tertiary structure observed here did not indicate whether antibody activity is due primarily to the tertiary structure at the antibody combining sites or due to the primary structure at these sites, the differences in tertiary structure being simply a reflection of differences in primary structure. The finding by Heidelberger *et al.* (29) which was confirmed by Green and Anker (30) that labeled γ-globulins passively infused into rabbits are not directly modified into antibodies does *not*, as has been suggested (31), preclude the possibility that antibody specificity resides in tertiary structure. It is possible, since antibodies are synthesized intracellularly, that a specific tertiary structure is formed only immediately after synthesis of the polypeptide chain; the failure to synthesize antibodies from circulating plasma γ-globulins might be attributable either to the failure of circulating γ-globulins to reenter the antibody synthesizing sites without being degraded in the cell or to an inability of the cell to unfold preformed γ-globulin to the extent required for subsequent antibody formation. Studies have revealed that almost all of the circulating γ-globulins thus infused enter the cells of the reticuloendothelial system, primarily those of the liver, where the protein is rapidly degraded (32, 33) and these are not the cells responsible for antibody synthesis (32, 34–36). On the other hand, it has been demonstrated by Smith *et al.* (25) that the amino acid composition of rabbit antibodies against Types I, VII, VIII, and XIV pneumococcus polysaccharides are very similar. It has also been demonstrated that the N-terminal sequence of five amino acids for a number of different rabbit antibodies and for normal rabbit γ-globulin is the same (37, 38). In view of the experimental errors inherent in amino acid analyses of proteins as large as the γ-globulins, however, these findings cannot be construed as indicating that different antibodies have the same primary structure.

Of the three concepts of antibody formation described in the introduction to this report, the data appear to be incompatible with the view that the primary

structure for all antibodies is identical and lends weight to the possibility that there may be an underlying genetic basis for antibody synthesis. Peptide differences among the different antibodies, however, were observed not only in those parts of the molecule associated with the combining sites for antigen but also in those parts of the molecule which did not appear to combine with antigen. The latter finding was surprising in view of the crystallizability of this non-combining polypeptide. In addition, more than one peptide difference appeared to be present between two different antibodies. These observations do not favor the concept that antibodies arise as the result of a single genetic mutation and suggest that different antibodies may arise in different groups of cells which had been genetically modified prior to antigenic stimulation although antigenic stimulation might induce further modification.

SUMMARY

Rabbit antibodies against pneumococcus capsular polysaccharides of Types II, III, VI, VII, and XII were isolated from type-specific antisera by precipitation with homologous antigen and hydrolyzed with either subtilisin, chymotrypsin or trypsin. Purified antibodies were also hydrolyzed with papain; the two polypeptides with active antibody sites and the crystallizable polypeptide were separated, studied in the ultracentrifuge and hydrolyzed with either subtilisin or chymotrypsin. The resulting peptides were separated on filter paper by electrophoresis and chromatography.

1. The two polypeptides with antigen-combining activity, fractions I and II, each constituted about 25 per cent of the original antibody molecule; the crystallizable polypeptide did not combine with antigen, fraction III, and represented about 35 per cent of the original antibody molecule. About 10 to 15 per cent of the original antibody molecule was hydrolyzed by papain into about 35 peptides.

2. The peptide patterns obtained for hydrolyzates of fraction I were almost identical with those obtained for fraction II obtained from the same antibody and were quite different for those obtained for fraction III.

3. Many of the peptide spots in the patterns obtained with whole antibody hydrolysates contained at least two peptides derived from different parts of the antibody molecule.

4. Differences were observed in the peptide patterns for different antibodies that suggested the existence of differences in both primary and tertiary structures among these antibodies.

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EXPLANATION OF PLATES

In all two-dimensional peptide patterns, the direction of electrophoresis is parallel to the bottom of each figure with the anode to the right and chromatography was ascending toward the top of the figure; the origin is marked by a short black line at the bottom to the right of center and this line was 7 mm. long in the original patterns. Unless otherwise stated, the patterns were developed with ninhydrin.

PLATE 22

FIGS. 1 to 6. Specific rabbit antibodies hydrolyzed with subtilisin.

FIG. 1. Antibodies against Type VI polysaccharide; unheated specific precipitate. Arrows at *a* should be compared with analogous area in the peptide pattern of Fig. 2 and the arrows at *b* with analogous areas in Figs. 2 to 4.

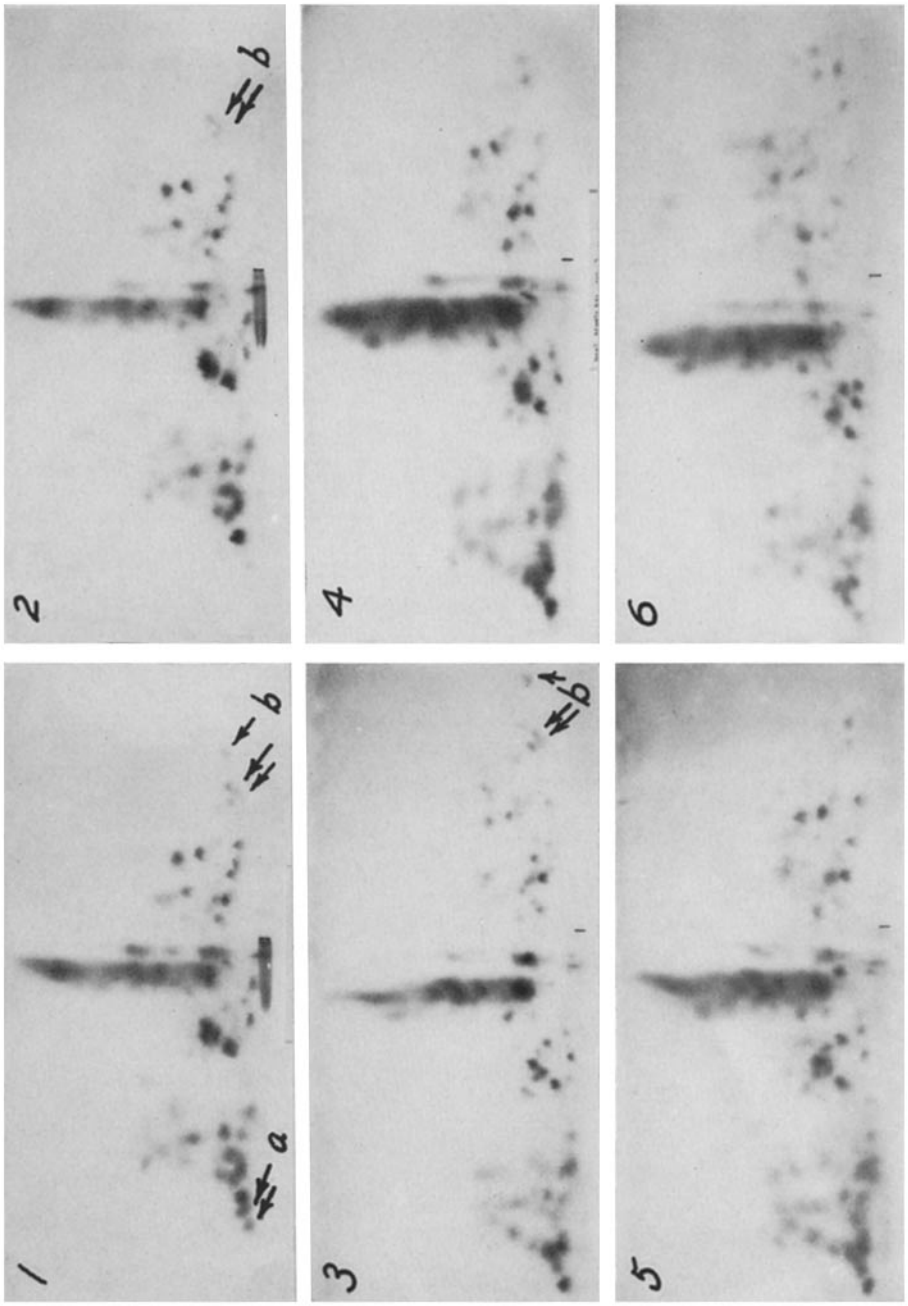
FIG. 2. Antibodies against Type VII polysaccharide; unheated specific precipitate. One of the two peptides indicated by arrow *a* in Fig. 1 could not be detected.

FIG. 3. Antibodies against Type II polysaccharide heated to 100°C prior to hydrolysis.

FIG. 4. Antibodies against Type VII polysaccharide heated to 100°C prior to hydrolysis. The peptides at *b* in Figs. 1 to 3 were absent.

FIG. 5. Performic acid oxidized antibodies against Type VI polysaccharide.

FIG. 6. Performic acid oxidized antibodies against Type VII polysaccharide.



(Gitlin and Merler: Peptides released by enzymatic hydrolysis)

PLATE 23

FIGS. 7 to 9. Specific rabbit antibodies oxidized with performic acid and hydrolyzed with chymotrypsin.

FIG. 7. Antibodies *vs.* Type VII polysaccharide.

FIG. 8. Antibodies *vs.* Type VII polysaccharide; pattern developed with Cl_2 followed by KI in starch.

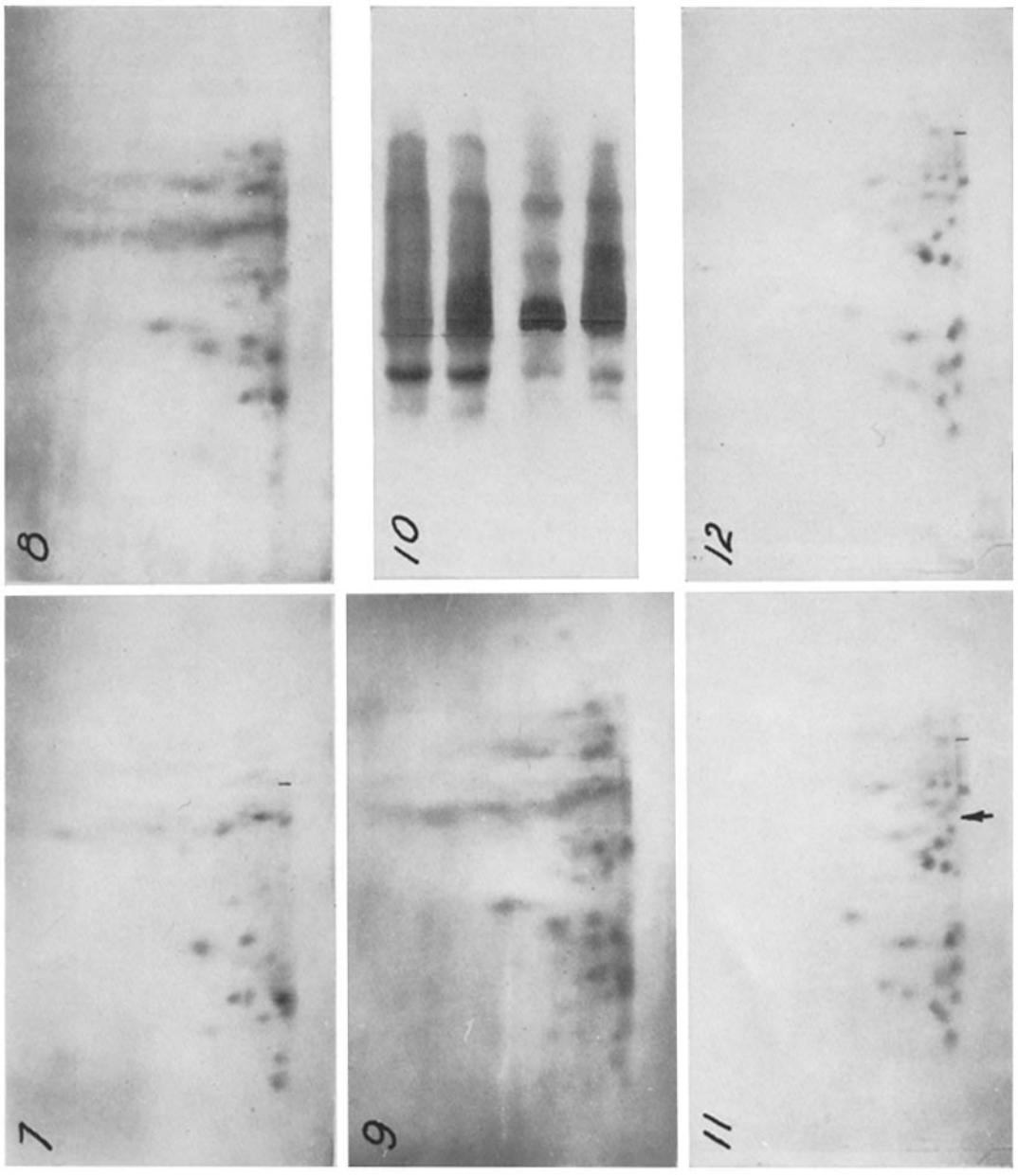
FIG. 9. Antibodies *vs.* Type VI polysaccharide; pattern developed with Cl_2 followed by KI in starch.

FIGS. 10 to 12. Antibodies oxidized with performic acid and hydrolyzed with trypsin.

FIG. 10. Fractions of hydrolysates insoluble at pH 4.2 electrophoresed at pH 8.0. Top to bottom: R_2 from anti-VII antibodies, R_2 from anti-VI antibodies, R_1 from anti-VII antibodies, and R_1 from anti-VI antibodies. Peptides detected with Cl_2 followed by KI in starch.

FIG. 11. Antibodies *vs.* Type VII polysaccharide.

FIG. 12. Antibodies *vs.* Type VI polysaccharide. Although the peptide indicated by arrow in Fig. 11 was not detected in this particular pattern, it was present in others prepared from the same hydrolysate and emphasizes that caution is necessary in interpreting differences between peptide patterns from different antibodies.



(Gitlin and Merler: Peptides released by enzymatic hydrolysis)

PLATE 24

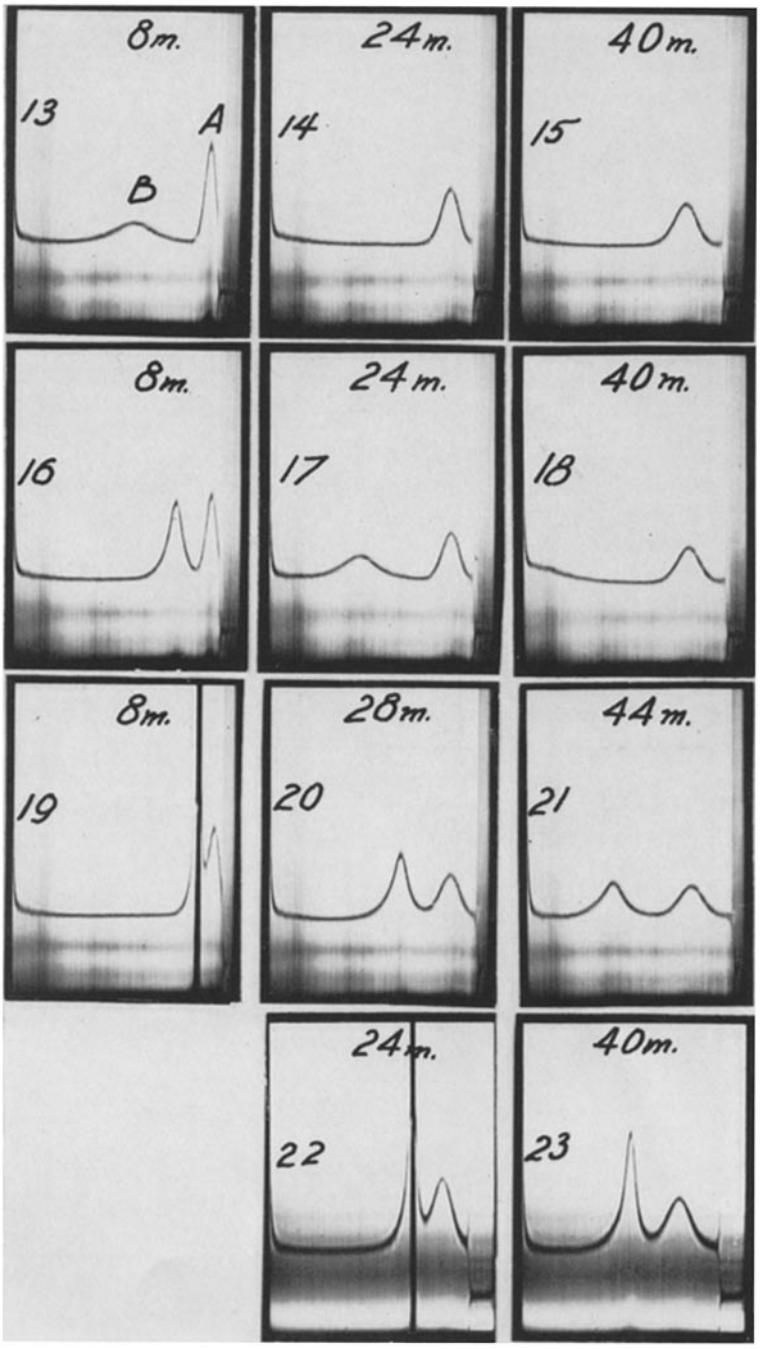
FIGS. 13 to 23. Ultracentrifuge patterns of antibodies against Type VII polysaccharide which were digested with papain and dialyzed and to which various amounts of homologous antigen were added; the antibody:antigen molecular ratio was calculated on the basis of amount of original antibody prior to hydrolysis. The time after reaching speed at which each frame was taken is indicated in minutes at the top center of each figure. The bottom of the cell is to the left of each frame.

FIGS. 13 to 15. Molecular ratio of antibody to antigen = 22. Component A is fraction III; component B is fraction I plus fraction II.

FIGS. 16 to 18. Molecular ratio of antibody to antigen = 8.7.

FIGS. 19 to 21. Molecular ratio of antibody to antigen = 4.5.

FIGS. 22 and 23. Molecular ratio of antibody to antigen = 1.6.



(Gitlin and Merler: Peptides released by enzymatic hydrolysis)

PLATE 25

FIGS. 24 to 31. Polypeptide fractions obtained from rabbit antibodies by hydrolysis with papain. The fractions were oxidized with performic acid and digested with subtilisin.

FIG. 24. Electrophoresis of fraction I from (top to bottom) anti-XII antibodies, γ-globulin from anti-VII antisera, and anti-VII antibodies. Arrows indicate some points of difference between Types XII and VII antibodies.

FIG. 25. Fraction I from anti-VII antibodies. Compare peptides at arrows with analogous area in Fig. 27.

FIG. 26. Fraction II from anti-VI antibodies.

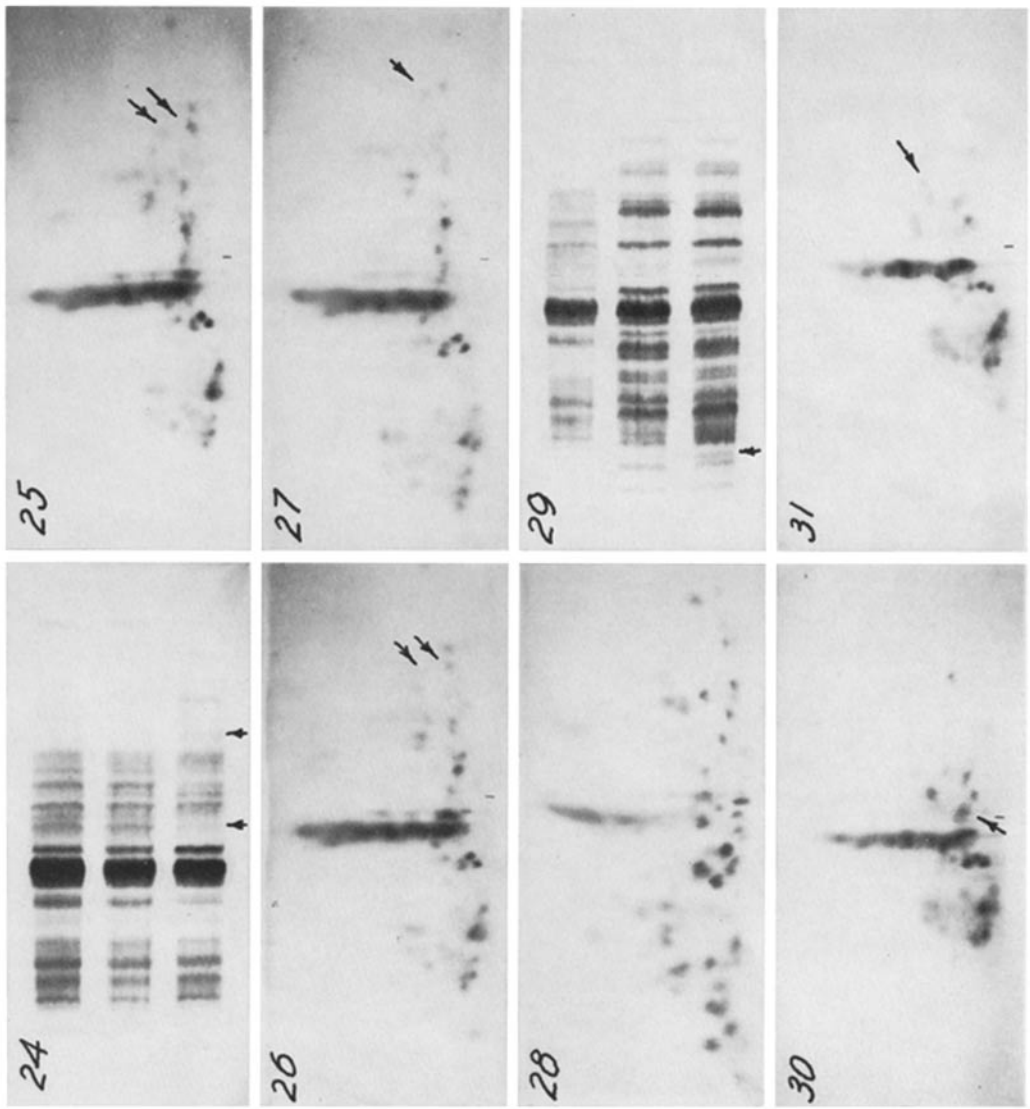
FIG. 27. Fraction I from anti-XII antibodies. Peptide at upper arrow of Figs. 25 and 26 is absent.

FIG. 28. Fraction III from anti-VII antibodies.

FIG. 29. From top to bottom: Fraction I from anti-XII antibodies, fraction III from anti-XII antibodies, fraction III from anti-VII antibodies.

FIG. 30. Fraction I from γ-globulin remaining in anti-VII antisera after removal of Type VII antibodies. See Fig. 40 and compare with Figs. 31 and 41. From area indicated by arrow, tyrosine, alanine, histidine, glutamic acid, aspartic acid, lysine, and proline were isolated; from analogous area of pattern in Fig. 31, all but proline were found.

FIG. 31. Fraction I from anti-VII antibodies. Area indicated by arrow contained valine, cysteine, proline, histidine, glutamic acid, aspartic acid, lysine, and glycine; analogous area in pattern of Fig. 30 lacked the cysteine and glutamic acid.



(Gitlin and Merler: Peptides released by enzymatic hydrolysis)

PLATE 26

FIG. 32. Fraction D from anti-VII antibodies released by papain.

FIGS. 33 to 39: Polypeptide fractions obtained from rabbit antibodies by hydrolysis with papain. The fractions were oxidized with performic acid and digested with chymotrypsin.

FIG. 33. Top to bottom: Fraction I from anti-XII antibodies, from γ-globulin of Type VII antiserum, from anti-VII antibodies.

FIG. 34. Fraction I from anti-VII antibodies. Compare peptides in areas indicated by larger two arrows with analogous areas in Fig. 35.

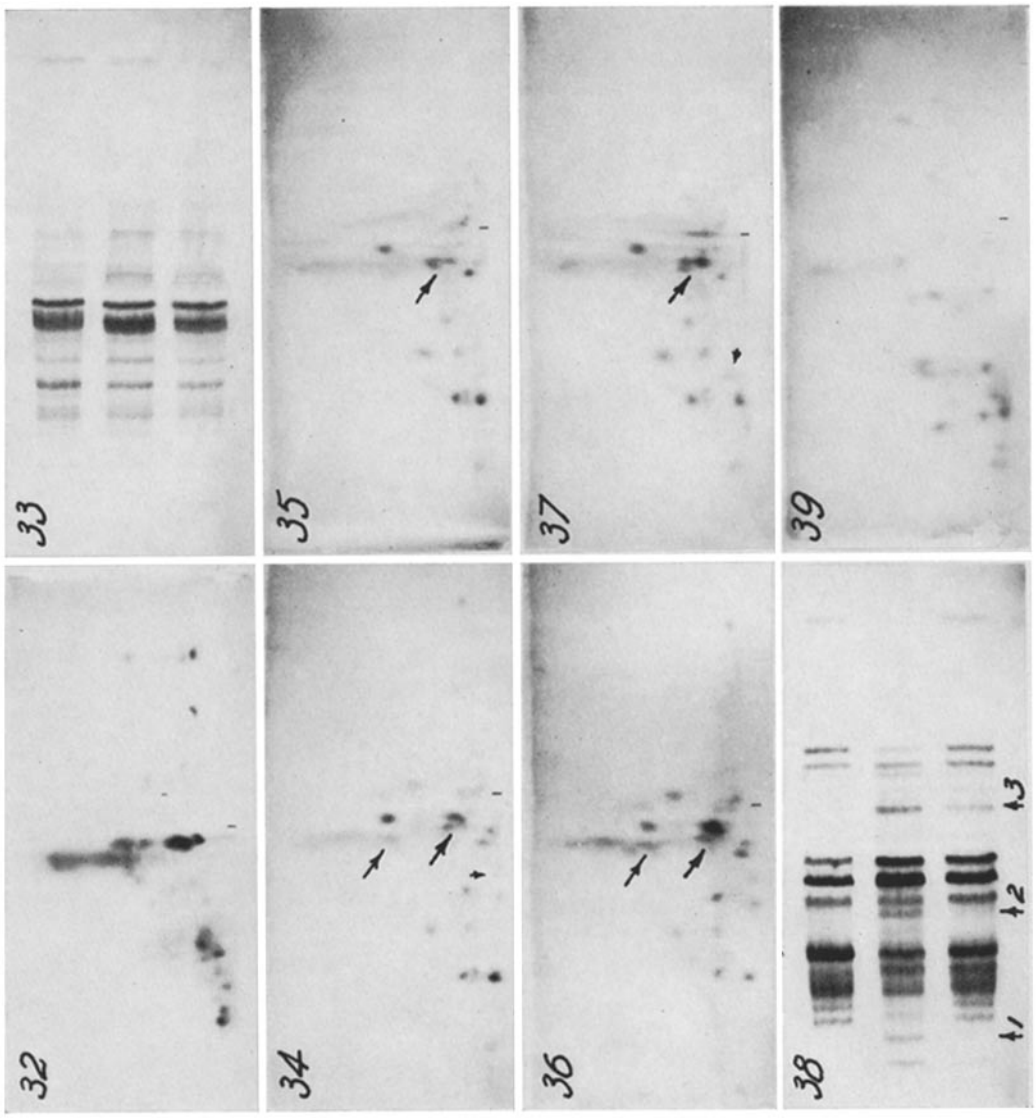
FIG. 35. Fraction I from anti-XII antibodies. Area indicated by arrow differs from analogous area in Fig. 34 and peptide at upper arrow of Fig. 34 is absent.

FIG. 36. Fraction II from anti-VII antibodies.

FIG. 37. Fraction II from anti-XII antibodies.

FIG. 38. Fraction III. Top to bottom: anti-XII antibodies, anti-VII antibodies, γ-globulin from Type VII antisera. Peptides in anti-VII antibodies at arrow 1 contained lysine not present in same area in anti-XII antibodies; at arrow 2, anti-VII contained tryptophane whereas anti-XII had glycine instead and at arrow 3, anti-VII appeared to have phenylalanine and anti-XII did not.

FIG. 39. Fraction III from anti-XII antibodies.

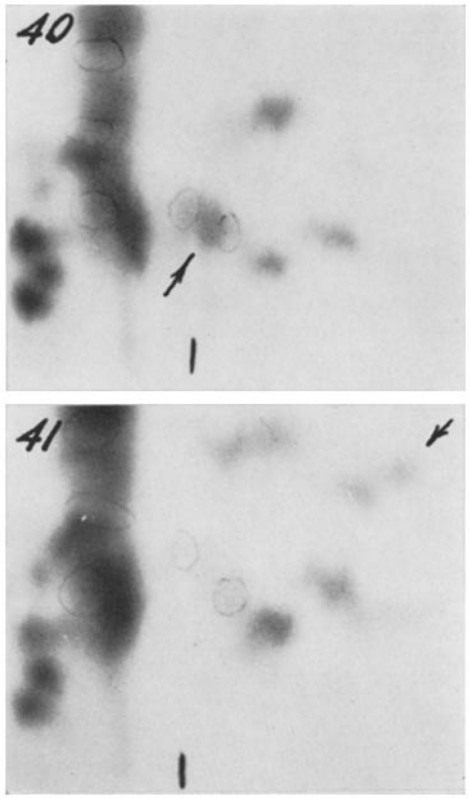


(Gitlin and Merler: Peptides released by enzymatic hydrolysis)

PLATE 27

FIG. 40. An enlargement of the area indicated by arrow in Fig. 30. The circles outline peptides that gave a yellow color with ninhydrin and were not readily reproduced in the photograph.

FIG. 41. An enlargement of the area indicated by arrow in Fig. 31. Compare with Fig. 40.



(Gitlin and Merler: Peptides released by enzymatic hydrolysis)