Binding of Univalent Antibody Fragments to a Distinct Antigenic Determinant of Staphylococcal Nuclease†

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ABSTRACT: Conditions for the production and purification of Fab fragments from goat γG antibodies have been determined and Fab fragments of antibodies to a distinct antigenic determinant in the region (99–126) of staphylococcal nuclease have been prepared. The reaction of these fragments with nuclease to form a soluble, enzymatically inactive complex has been followed spectrophotometrically using the rate of cleavage of substrate DNA as a measure of free, residual nuclease concentration, and an equilibrium constant for the reaction has been determined. The Scatchard plot of the equilibrium binding data shows a sharp bend at half-saturation of antibody binding sites similar to that previously reported for intact, divalent anti-$(99–126)_N$ antibodies. Since interaction between binding sites should not be possible for univalent Fab fragments, we interpret this result to indicate the presence of approximately equal amounts of two antibody populations within anti-$(99–126)_N$, one of average $K_{ass} \sim 10^9\ M^{-1}$, and the second of average $K_{ass} \sim 10^7\ M^{-1}$. This conclusion is supported by isoelectric focusing experiments, which show that the sequential fractionation of anti-nuclease antibodies to produce the anti-$(99–126)_N$ population is attended by progressive restriction of antibody heterogeneity, with, however, at least two distinct species remaining even at the final stage.

We have recently reported the preparation of a population of antibodies specific for an antigenic determinant in the polypeptide chain between amino acids 99 and 126 of staphylococcal nuclease (nuclease)[1] (Sachs *et al.*, 1972a). Kinetic and equilibrium constants describing the inactivation of nuclease by this specific antibody preparation have also been reported (Sachs *et al.*, 1972b). The Scatchard plot of the equilibrium binding data was found to bend sharply in the region $r = 1$, yielding two average association constants. Two possible explanations for these results were offered: first, that there might be two subpopulations of antibody present in the anti-$(99–126)_N$;[2] second, that the binding of a molecule of nuclease to one binding site made energetically unfavorable the binding of a second molecule of nuclease. This paper presents studies undertaken to distinguish between these two possibilities employing Fab fragments of anti-$(99–126)_N$ antibodies.

Materials and Methods

Nuclease was prepared and purified by published methods (Moravek *et al.*, 1969; Cuatrecasas *et al.*, 1968). Goat anti-nuclease antiserum was prepared and fractionated into subpopulations by immunoadsorption as previously described (Sachs *et al.*, 1972a).

Preparation of Fab Fragments of Anti-$(99–126)_N$. (A) Papain. Anti-$(1–149)_N$ antibodies (500 mg) were digested with papain at 37° for 24 hr in 0.1 M sodium phosphate (pH 7.0) containing 0.02 M cysteine and 0.01 M EDTA, at an enzyme to protein ratio of 1:25 (w/w), in a manner similar to that described by Porter (1959). After dialysis and centrifugation the material was chromatographed in phosphate buffered saline (0.13 M NaCl–0.015 M Na_2HPO_4–0.004 M KH_2PO_4) on Sephadex G-75, and then successively adsorbed to and eluted from Sepharose–(99–149)[3] and Sepharose–(127–149) under the same conditions used previously to prepare anti-$(99–126)_N$ antibodies (Sachs *et al.*, 1972a) (see Figure 1).

(B) Trypsin. Anti-$(1–149)_N$ (200 mg) was digested with trypsin in 20 ml of 0.1 M Tris-HCl (pH 8.1) containing 0.02 M $CaCl_2$ at 37° at a final enzyme to protein ratio of 1:25 (w/w). Equal aliquots of trypsin (4 mg) were added at 0 and 3 hr. After 6 hr the digest was centrifuged and chromatographed on a column of Sephadex G-150 (2.8 × 30 cm) in 0.15 M NaCl (Underdown *et al.*, 1971). As described below this digestion produced mainly Fab'_2 fragments, as indicated by their apparent molecular weight by Sephadex G-150 chromatography (Figure 2). These divalent fragments were successively adsorbed to and eluted from Sepharose–(99–149) and Sepharose–(127–149), as shown in Figure 3. The effluent of unbound material from the second column, consisting of anti-$(99–126)_N$ Fab'_2 fragments, was treated with 0.005 M dithiothreitol in 0.1 M Tris-HCl (pH 8.1) for 40 min at 25°, and then iodoacetamide was added to a final concentration of 0.01 M. After a further incubation for 15 min the mixture was chromatographed on Sephadex G-150 in 0.15 M NaCl. The major peak consisted of fragments with a molecular weight which indicated complete reduction to Fab fragments.

Equilibrium inactivation studies were performed as previously described (Sachs *et al.*, 1972b). After incubation for 15 min at 25° aliquots were removed from the reaction mixture

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[1] Staphylococcal nuclease is an enzyme consisting of 149 amino acids whose amino acid sequence (Bohnert and Taniuchi, 1972) and three-dimensional structure (Cotton and Hazen, 1971) are known.

[2] Anti-$(99–126)_N$ refers to antibodies directed against region 99–126 of staphylococcal nuclease and obtained by fractionation of anti-nuclease antiserum on columns of Sepharose to which selected fragments of nuclease had been covalently attached. The subscript N indicates that the antibodies were obtained by immunization with native nuclease and are therefore presumably directed against the native conformation of this sequence in the enzyme. The subscript N replaces n as used in our previous reports in order to conform with the literature of protein denaturation (Tanford, 1970). Although fragment 1–149 is actually intact nuclease rather than a nuclease fragment, the numerical designation has been used for consistency.

[3] Sepharose–(99–149) refers to Sepharose 4B to which the nuclease fragment containing amino acid residues 99–149 has been covalently bound.

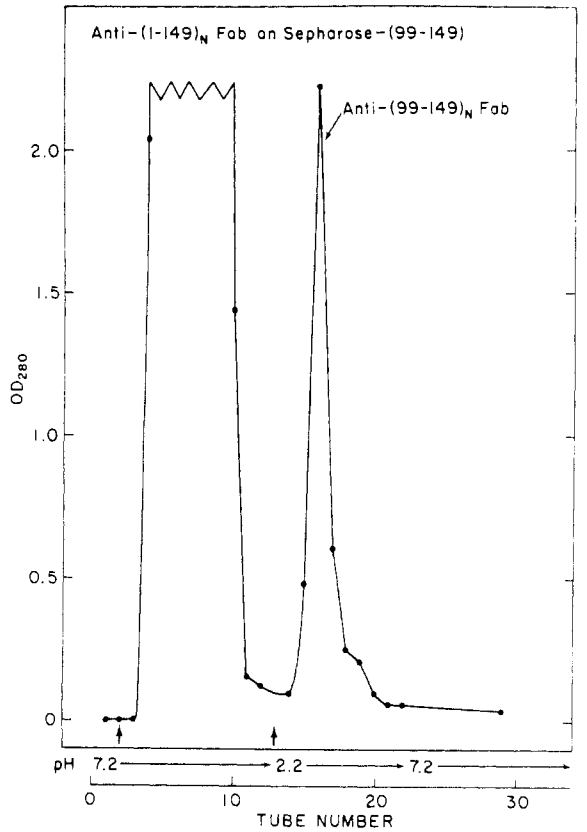

FIGURE 1: Affinity chromatography on Sepharose-(99–149) of anti-(1–149)$_N$ Fab fragments, produced by papain digestion. The first arrow indicates the addition of the sample. At the second arrow the buffer was changed to pH 2.2 Sorenson's citrate and a peak of inactive Fab fragments of anti-(99–149)$_N$ eluted (see Results).

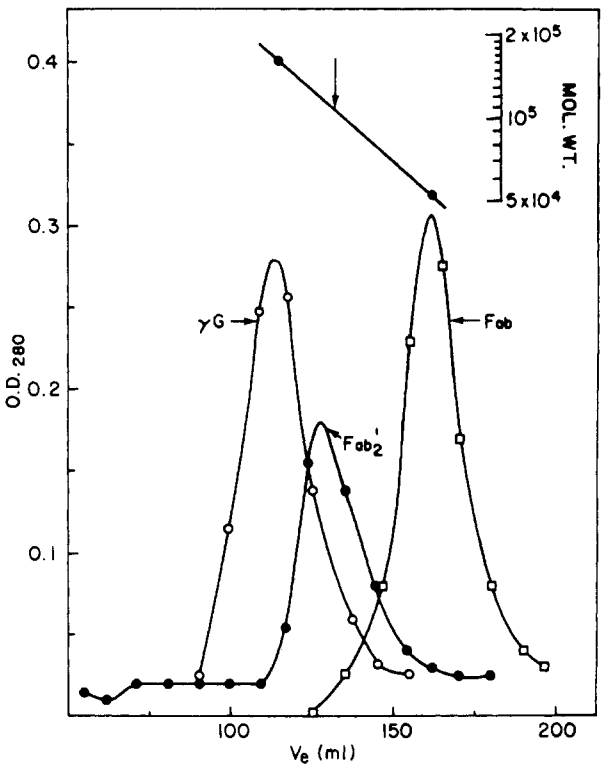

FIGURE 2: Composite results of chromatography on Sephadex G-150 of anti-(1–149)$_N$ (○), Fab fragments of anti-(1–149)$_N$ prepared by papain digestion (□), and the product of trypsin digestion of anti-(1–149)$_N$ (●). The molecular weight of the trypsin digestion product was estimated on the basis of the elution positions and known molecular weights of the γG, and Fab fragments produced in the standard fashion with papain, assuming a linear relationship, between elution volume and molecular weight in this limited region (Andrews, 1965). The inset shows this molecular weight *vs.* elution volume relationship.

and assayed for residual nuclease activity by the standard spectrophotometric assay (Cuatrecasas *et al.*, 1967). The data were analyzed both by plotting nuclease activity as a function of nuclease concentration, and according to the method of Scatchard (1949) as previously described for intact anti-(99–126)$_N$ antibodies (Sachs *et al.*, 1972b).

Isoelectric focusing of intact γG was performed at 4° according to the method of Doerr and Chrambach (1971). Antibody samples were dialyzed against pH 8–10 Ampholine (LKB) and focused through 4% acrylamide gels containing pH 5–8 Ampholine for 18 hr at a constant voltage of 200 V. The upper buffer compartment (cathode) was filled with 0.4% ethylenediamine, and the lower buffer reservoir (anode) was filled with 0.02% sulfuric acid. The gels were stained with 0.1% Coomassie Blue and destained according to the method of Vesterberg (1971). The pH gradient was determined by running one gel without protein. It was then sliced into 45, 1-mm pieces, and each was kept overnight in 1 ml of 0.03 M KCl in a CO_2-free atmosphere before its pH was determined.

Results

Preparation and Selective Fractionation of Univalent Anti-(99–126)$_N$ Antibodies. Since immunoadsorption provides a rapid and efficient method of purifying antibodies from other contaminating materials, our plan was first to digest the divalent antibody population anti-(1–149)$_N$ to Fab fragments and then to fractionate those fragments successively on columns of Sepharose-(99–149) and Sepharose-(127–149), as previously described for the fractionation of the intact antibody populations (Sachs *et al.*, 1972a). In addition to its usefulness in removing extraneous digestion products, such a functional purification would assure the removal of denatured or modified antibody fragments which might otherwise interfere with determination in subsequent binding studies of the effective combining site concentration.

Digestion of the anti-nuclease γG with papain produced functional Fab fragments as indicated by the ability of these fragments to bind to Sepharose-(99–149) (Figure 1). However, the Fab fragments, when eluted in 0.1 N Sorenson's citrate (pH 2.2), were found to have lost all of their ability to inactivate nuclease. Presumably, the reductive milieu which is necessary for papain digestion produced Fab fragments lacking disulfide bridges. It was reasoned that the acidic elution conditions may have destroyed the noncovalent interactions which held these fragments together, leading to loss of antibody activity in the eluted peak. This hypothesis was confirmed by Sephadex G-200 tlc in pH 2.2 Sorenson's citrate buffer which showed a decrease in the molecular weight of these fragments as compared to a similar thin-layer chromatogram (tlc) at pH 7.2 (Johansson and Rymo, 1964). Cleavage of anti-nuclease γG with trypsin, a method not requiring reducing conditions, was therefore attempted.

Digestions of mouse and human γG antibodies with trypsin have previously been shown to yield mainly Fab fragments (Underdown *et al.*, 1971; Schrohenloher, 1963). Using similar digestion conditions we have found the major product of digestion of goat anti-nuclease antibodies to elute from a Sephadex G-150 column in a region corresponding to a molecular weight of 105,000 (Figure 2). Thus, presumably, trypsin

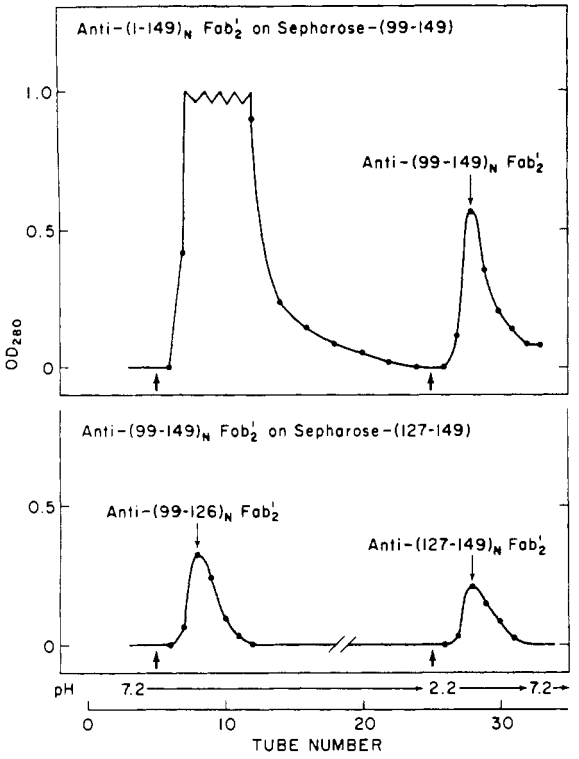

FIGURE 3: Affinity chromatography of Fab'$_2$ fragments of anti-(1–149)$_N$ on Sepharose–(99–149) and Sepharose–(127–149). The material which bound to and was eluted from Sepharose–(99–149) was applied to Sepharose–(127–169) after neutralization and concentration to 5 ml in 0.15 M NaCl. (The position of the second peak on the elution pattern of Sepharose–(127–149) has been displaced ten tubes to the right for clarity of presentation.)

cleavage of goat γG leads to Fab'$_2$ fragments as the major product, in contrast to the production of Fab fragments by the similar digestion of mouse or human γ-globulin. This probably reflects structural differences between the γ-globulins of these species, such as the relative positions of the interchain disulfides between Fab regions (Edelman and Gall, 1969).

Unlike the Fab fragments produced by papain digestion, the Fab'$_2$ fragments produced by trypsin digestion retained full antibody activity after elution from Sepharose–(99–149) in pH 2.2 citrate buffer. Purification by sequential immunoadsorption could thus be carried out in the same fashion as had been reported for intact γ-globulin (Sachs *et al.*, 1972a). At the final stage of this purification anti-(99–126)$_N$ Fab'$_2$ fragments were reduced and alkylated, and the resulting Fab fragments, after purification by gel filtration, retained full activity.

Equilibrium Studies. The mean activities of aliquots of mixtures of Fab fragments of anti-(99–126)$_N$ with increasing concentrations of nuclease are plotted in Figure 4. The activities of aliquots of mixtures containing the same nuclease concentrations in the absence of antibody are plotted for comparison. When the linear portion of the curve of nuclease activity *vs.* nuclease concentration in the presence of antibody is extrapolated to zero activity it intersects the abscissa at a nuclease concentration of 7.5×10^{-8} M, approximately equal to one-half the concentration of the Fab fragments used in these studies. A Scatchard plot of the equilibrium data from several experiments is shown in Figure 5. Because of the limited accuracy of assays at low free enzyme concentrations, the data for the Scatchard analysis are restricted. However, the Scatchard plot can be interpreted as indicating that half the antibody sites bind nuclease with an average affinity of

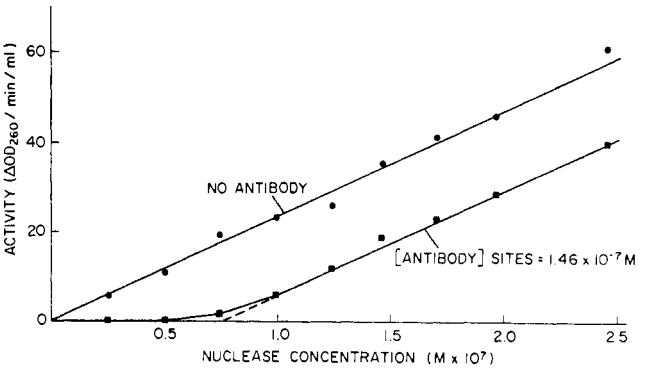

FIGURE 4: Equilibrium study of nuclease inactivation by Fab fragments of anti-(99–126)$_N$ produced by trypsin digestion. Enzymatic activity is plotted against nuclease concentration in the presence of 1.46×10^{-6} M Fab fragments (●) and in the absence of Fab fragments (■) (average of several experiments). The dashed line, an extrapolation to the point of equivalence for a high affinity species, intersects the *x* axis at a nuclease concentration of 7.5×10^{-8} M.

about 3×10^9 M^{-1} and that the other half bind with an average affinity of about 1×10^7 M^{-1}.

Isoelectric Focusing of Intact γG. In an effort to determine a possible structural basis for such functional heterogeneity, purified γG preparations were subjected to isoelectric focusing (Figure 6). Between pH 5 and 8 purified anti-(1–149)$_N$ γG showed seven bands of Coomassie Blue stained material, of which at least five clearly represented regions of multiple, unresolved species. This, nevertheless represents restriction of heterogeneity with respect to non-immune γG (Awdeh and Williamson, 1970). By comparison, the pattern of anti-(99–149)$_N$ showed absence or diminution of at least two of these bands. Separation of anti-(99–149)$_N$ into component populations of anti-(99–126)$_N$ and anti-(127–149)$_N$ (Sachs *et al.*, 1972a) showed that some of these bands were unique to one or the other of these two populations. Anti-(99–126)$_N$ contained primarily bands with p*I* values from 6.0 to 6.4, while anti-(127–149)$_N$ contained the more cathodal bands with p*I* values from 6.2 to 6.8. The central, broad band of anti-(1–149)$_N$ and anti-(99–149)$_N$ appears to be resolved into three-component

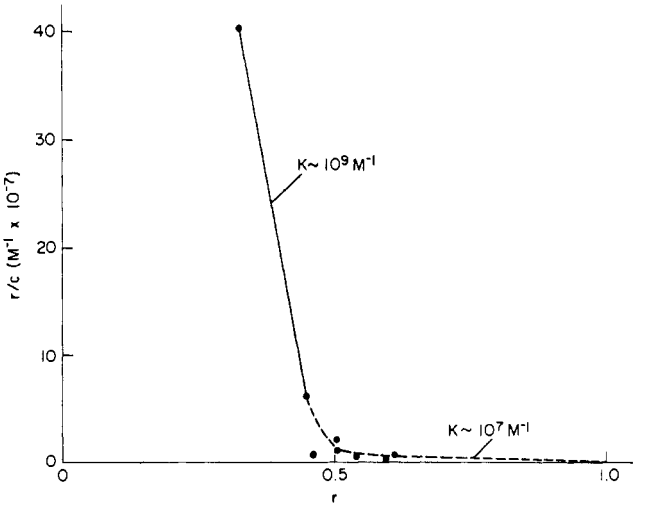

FIGURE 5: Scatchard analysis of the data from several equilibrium experiments of the inactivation of nuclease activity by Fab fragments of anti-(99–126)$_N$. The dashed portion of the curve has been drawn so as to intersect the abscissa at $r = 1$ in accordance with known values for Fab fragments of γG immunoglobulins.

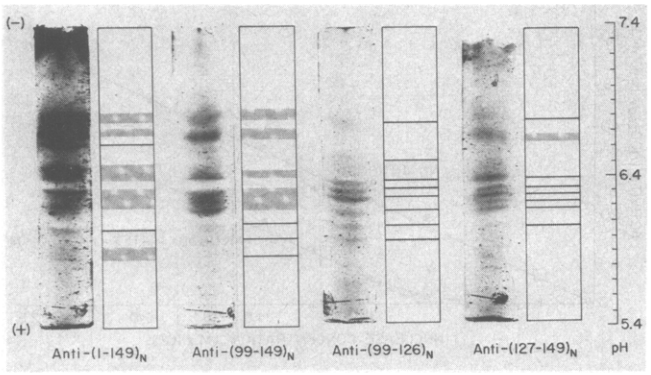

FIGURE 6: Isoelectric focusing of 40 μg of anti-$(1–149)_N$, 40 μg of anti-$(99–149)_N$, 25 μg of anti-$(99–126)_N$, and 25 μg of anti-$(127–149)_N$. The linear pH gradient established in the gels is shown on the right. A line drawing of the observed bands is shown to the right of each gel for clarity.

and four-component species in anti-$(99–126)_N$ and anti-$(127–149)_N$, respectively.

The appearance of isoelectric patterns with several bands spaced at regular intervals has been interpreted as consistent with a monoclonal γG product which has undergone post-translational alteration of charge (Askonas *et al.*, 1970). The two triplets of bands of approximately equal intensity seen in anti-$(99–126)_N$ would be consistent with two primary species of antibodies to this limited region of nuclease.

Discussion

Anti-$(99–126)_N$ is an operationally monospecific antibody population directed against an antigenic determinant in the region 99–126 of the native nuclease molecule. Because the production of a precipitating lattice network depends on the presence of multiple specificities (Marrack *et al.*, 1951) in an antibody population, this anti-$(99–126)_N$ population does not cause precipitation with nuclease. It does, however, bind to nuclease as determined by its capacity to inactivate the antigen's enzymatic activity. This system has therefore lent itself to kinetic and equilibrium studies of the interaction of nuclease and antibody in solution (Sachs *et al.*, 1972b). In those studies we noted the anomalous finding that the binding of nuclease by antibody to form an inactive complex occurred only up to a concentration of nuclease just equal to the antibody concentration. The binding was anomalous in that each antibody molecule ought to be capable of binding two molecules of antigen, producing complete inactivation up to a concentration of nuclease twice that of antibody. This discrepancy was also reflected in the Scatchard plot of data from several equilibrium experiments, which bends sharply at $r = 1$, yielding two average association constants differing by almost two orders of magnitude.

As mentioned earlier, two possible explanations for these results have been offered (Sachs *et al.*, 1972b). One postulates that two populations of antibodies exist in anti-$(99–126)_N$. One population has an average $K_{ass} = 8.3 \times 10^8\ M^{-1}$, and the other has an average $K_{ass} = 1.7 \times 10^7\ M^{-1}$; each population represents one-half of the antibody molecules present. In this case the nearly complete inactivation of nuclease up to a concentration equal to half that of antibody would be due to the higher affinity antibody.

The other possible explanation is that although all the antibody binding sites for nuclease are equivalent, the binding of one molecule of nuclease makes energetically unfavorable (negative cooperativity) the binding of the second molecule of nuclease. While such interference has not previously been described, we thought this explanation not unlikely considering the much larger size of nuclease compared to the haptens generally used in equilibrium binding studies (Eisen and Siskind, 1964). The simplest approach to the question appeared to be preparation of monovalent Fab fragments of anti-$(99–126)_N$. Since no direct interaction would be possible for such fragments, equilibrium binding studies would be expected to distinguish between the two proposed explanations.

We have reported the details of our experience in the production of functional Fab fragments because of the differences we have noted between the behavior of goat and mouse or human γG immunoglobulins and because, to our knowledge, methods for preparing goat Fab fragments by trypsin digestion have not previously been reported.

Our experience with papain digestion illustrates the necessity for considering the lability of reduced Fab fragments in designing methods for purification and handling. Confining purification by immunoadsorbants to the Fab'_2 stage of the preparation would seem to be an effective way to make use of this powerful fractionation technique while avoiding losses of activity which could attend similar procedures after reductive alkylation. Alternatively it may be possible to control conditions of reoxidation in order to regenerate specific antigen binding activity in reduced and denatured Fab fragments. However, in the case of rabbit antibody fragments only partial recovery of activity has been obtained by this method (Haber, 1964).

The equilibrium inactivation data shown in Figure 4 are almost identical with those previously reported for the reaction between nuclease and intact anti-$(99–126)_N$ antibodies; *i.e.*, inactivation was complete up to a concentration of nuclease equal to half the concentration of antibody combining sites. The Scatchard analysis (Figure 5), although of low precision because of the limited range of concentrations in the region of partial saturation, is similar to that which we have previously published for the intact immunoglobulin (Sachs *et al.*, 1972b). The points can likewise be fitted by assuming two equal populations of combining sites with average association constants of about 10^9 and $10^7\ M^{-1}$. These data thus indicate that negative cooperativity in the binding of a second molecule of nuclease to a divalent molecule of anti-$(99–126)_N$ cannot explain our results, since if this explanation were correct we would expect the Scatchard plot of our equilibrium inactivation data for the Fab fragments to show a straight line intersecting the abscissa at $r = 1$ indicating a single average association constant. The data are consistent, however, with the existence of two distinct subpopulations within anti-$(99–126)_N$, having average association constants differing by almost two orders of magnitude.

Heterogeneity of antibodies is the rule rather than the exception for Scatchard plots of antibody–antigen binding. However, most often the heterogeneity is characterized by a smooth curve indicating a continuum of antibody affinity constants (Kabat, 1961), rather than by a curve with a sharp bend such as that of Figure 5. The fact that just half of the antibodies fall into each of the two subpopulations may not be merely fortuitous. It may indicate, for example, that distinct antigenic determinants lead to the production of populations of antibodies of restricted heterogeneity. This has been demonstrated for antibodies directed against a limited region, the "loop"-peptide, of lysozyme (Maron *et al.*, 1971).

Our isoelectric focusing data support this hypothesis. As seen in Figure 6 anti-$(1–149)_N$ showed considerable heterogeneity in banding patterns. However, this heterogeneity is

less than that observed in non-immune γG (Awdeh and Williamson, 1970). Each subsequent fractionation reduced the heterogeneity, and anti-$(99–126)_N$, the operationally monospecific population, had six major bands in two triplets and two minor bands. In accordance with the findings of Askonas *et al.* (1970), it is possible that the two triplets represent two distinct antibody species which have undergone post-translational modification. However, the correspondence between such restricted structural heterogeneity and the limited heterogeneity of binding affinities that we have observed are not yet known.

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