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Inactivation of Staphylococcal Nuclease by the Binding of Antibodies to a Distinct Antigenic Determinant†

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ABSTRACT: The interaction of antibodies with a distinct antigenic determinant in the region (99–126) of staphylococcal nuclease has been found to produce a soluble, inactive antibody–nuclease complex. The reaction has been followed spectrophotometrically, using the rate of hydrolysis of substrate DNA as a measure of residual, free nuclease concentration. This analysis has provided the following kinetic and equilibrium constants for the antibody–antigen interaction: $K_{ass} = 8.3 \times 10^8\ M^{-1}$, $k_{on} = 4.1 \times 10^5\ M^{-1}\ sec^{-1}$, $k_{off} = 4.9 \times 10^{-4}\ sec^{-1}$. Conversely, measurement of changes in nuclease activity can be used as a rapid and sensitive assay of antibody concentration. The Scatchard plot of the equilibrium inactivation data bends sharply near $r = 1$, which may reflect heterogeneity of the antibodies or may indicate possible steric interference in the binding of a second nuclease molecule to the bivalent antibody molecule.

We have recently reported the preparation of a population of antibodies specific for an antigenic determinant formed by amino acids in the region (99–126)[1] of staphylococcal nuclease (Sachs *et al.*, 1972). These antibodies, obtained by fractionation of goat anti-nuclease serum on immunoabsorbents bearing selected polypeptide fragments of nuclease, constituted approximately 3% of the total antibody of that serum. They have been designated as anti-$(99–126)_n$, in which the subscript, "*n*," indicates that the antibodies were obtained by immunization with the intact enzyme and are therefore presumably directed against the native conformation of this polypeptide sequence of nuclease.

The molecular location of this determinant is illustrated in Figure 1, which is an artist's representation of the crystallographic structure of nuclease in which the sequence (99–126) is darkened. Since DNA, the natural substrate of nuclease, is thought to fit into a "cleft" in the front of the molecule (Arnone *et al.*, 1971), as it is viewed in Figure 1, the presence of a bulky antibody molecule attached to a determinant in the region (99–126) might be expected to inhibit the enzyme's activity on DNA. Furthermore, because the component antibodies of anti-$(99–126)_n$ can combine with only a single site per nuclease molecule the resultant interaction does not lead to precipitation (Sachs *et al.*, 1972). Both of these considerations suggested the feasibility of a study of the combination of anti-$(99–126)_n$ with nuclease using the spectrophotometric assay of nuclease to monitor the interaction. We have found that the antibody does inhibit the enzymatic activity and this paper reports kinetic and equilibrium aspects of the antibody-induced inactivation.

Materials and Methods

Preparation and purification of nuclease, immunization procedures, and fractionation of the anti-nuclease serum have

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[1] Nuclease consists of a single polypeptide chain of 149 amino acids, numbered 1–149 from amino to carboxyl-terminal ends. 99–126 thus refers to a limited region of this polypeptide chain.

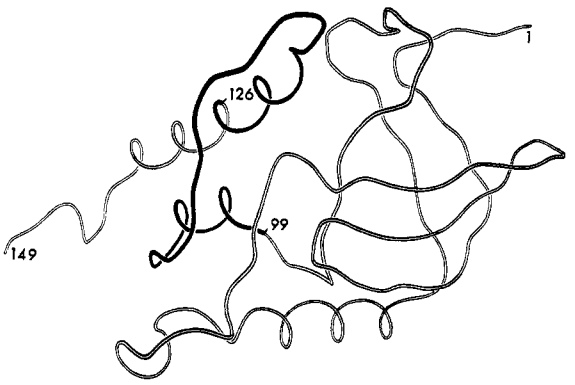

FIGURE 1: An artist's representation of the three-dimensional structure of staphylococcal nuclease. The drawing was made from a wire model, based on the X-ray crystallographic structure (Cotton and Hazen, 1971). The sequence between amino acids 99 and 126 has been shaded, indicating the molecular localization of the antigenic determinant of anti-$(99–126)_n$.

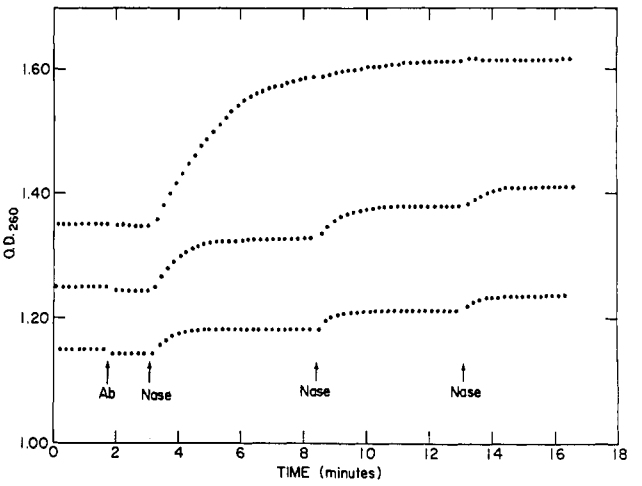

FIGURE 2: Inactivation of nuclease by anti-$(99–126)_n$. Three simultaneous activity assays are shown as recorded on a Gilford multiple sample absorbance recorder, with full scale of 1.0 OD unit at 260 nm. At the time indicated by the first arrow 0.00, 2.85, and 5.70 μg of antibody were added to the cuvets of the uppermost, middle, and lowermost assays, respectively. At the times indicated by subsequent arrows 0.05 μg of nuclease was added to each cuvet.

been described (Sachs *et al.*, 1972). The studies reported in this paper employed aliquots of a single preparation of anti-$(99–126)_n$ isolated from 20 ml of anti-nuclease serum.

Enzymatic assays of nuclease activity were performed on a Gilford Model 2000 multiple sample absorbance recorder equipped with a Beckman DU spectrophotometer using denatured DNA as the substrate and measuring changes in optical density at 260 nm, as described by Cuatrecasas *et al.* (1967). In order to minimize losses of nuclease by adsorption to glassware, 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was added to all dilutions of nuclease concentrations less than 1.0 mg/ml and such solutions were manipulated with siliconized glassware (Cuatrecasas *et al.*, 1967). In order to obtain reproducible absolute nuclease activities with different preparations of DNA substrate it was found helpful to use a nuclease standard and to correct all activity measurements with a particular substrate solution according to the absolute activity of this standard. The standard nuclease solution contained highly purified nuclease at a concentration of 5 μg/ml in 0.1% bovine serum albumin, and was frozen in small aliquots. A 10-μl sample of this standard produced a change of 0.072 OD_{260} unit/min with a freshly prepared substrate solution on the day of preparation of the standard. Subsequent values obtained for the activities of this standard ranged from 0.065 to 0.088 OD_{260} unit per min, probably reflecting differences in the degree of denaturation of individual DNA preparations. By multiplying the activity values obtained with any particular DNA preparation by the ratio of 0.072 to the change in absorption per minute caused by 10 μl of the standard, assayed on that DNA preparation, highly reproducible, absolute activity measurements were obtained.

Kinetic studies of antibody-induced inactivation of nuclease were performed at a constant temperature of 25° by perfusing the cuvet chamber with water from a constant-temperature bath (Haake). One milliliter of the substrate solution (50 μg of heat-denatured salmon sperm DNA/ml of 0.025 M Tris buffer, pH 8.8, 0.01 M in $CaCl_2$) was introduced into each cuvet. After stable base-line values at OD_{260} had been maintained for at least 2 min, the aliquot of antibody solution to be assayed was introduced into the substrate solution. The base-line values were observed for stability for another minute, and then nuclease was added to the cuvets and the change in OD_{260} was recorded in the usual fashion.

Equilibrium inactivation studies were performed by mixing reactants in the wells of siliconized microtiter U plates (Cooke Engineering Co.). The required volumes, from 10 to 100 μl of appropriate concentrations of nuclease in 0.1% bovine serum albumin, were added to wells containing anti-$(99–126)_n$ in 0.15 M NaCl and a volume of 0.1% bovine serum albumin sufficient to equalize the final volumes of all wells. After incubation of the plates at 25° for 15 min aliquots were removed and assayed for residual nuclease activity by the standard spectrophotometric assay. The attainment of equilibrium was confirmed by testing successive aliquots from the same well at 30 min and at 1 hr. The data were analyzed both directly by plotting activity as a function of nuclease concentration, and by the method of Scatchard (1949), according to eq 1, in which r represents the number of nuclease mole-

$$r/c = Kn - Kr \qquad (1)$$

cules bound per antibody molecule at a concentration of free nuclease equal to c, K is the association constant for the interaction, and n equals the maximum number of nuclease molecules that can be bound per antibody molecule.

Results

Kinetics of Inactivation. The introduction of anti-$(99–126)_n$ into the substrate solution was found to alter the pattern of the change in OD_{260} caused by the addition of nuclease. This effect is illustrated in Figure 2 which shows typical results obtained in spectrophotometric assays of nuclease (0.05μg) in the absence of antibody and in the presence of two concentrations of anti-$(99–126)_n$. The control assay with no added antibody (uppermost curve) showed a linear change of absorption over the first 2 min with a slope of 0.080 OD_{260} unit/min, corresponding to the expected activity of this amount of nuclease. After 2 min the slope decreased as substrate was exhausted. In the second assay 2.85 μg of anti-$(99–126)_n$ antibody was added to the cuvet before the assay was performed. While the slope was initially similar to that

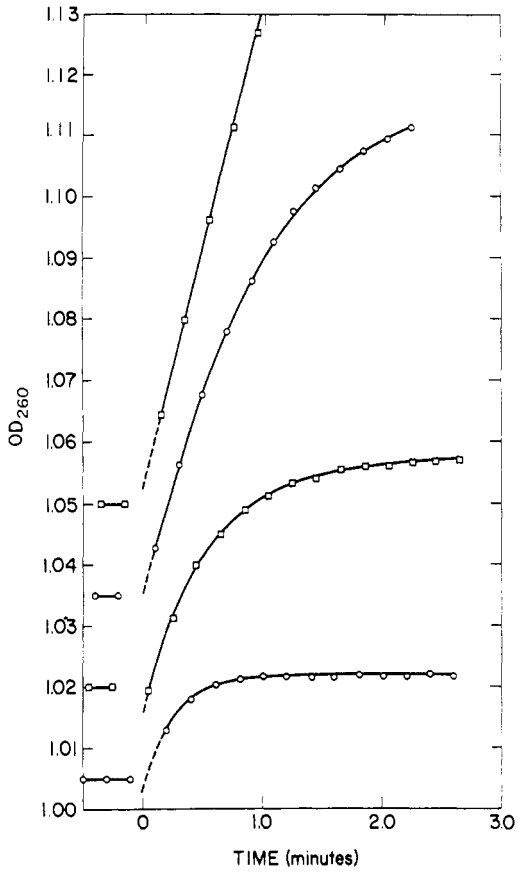

FIGURE 3: Effect of anti-$(99–126)_n$ on initial nuclease activity: These data were obtained in a manner similar to those of Figure 2, except that the recorder was adjusted to a full scale of 0.1 OD unit at 260 nm and the chart speed was increased. From top to bottom the recorded assays represent cuvets to which 0.00, 2.85, 5.70, and 11.40 μg of antibody were added before the addition of 0.05 μg of nuclease. The points were connected by smooth curves by hand to aid visualization.

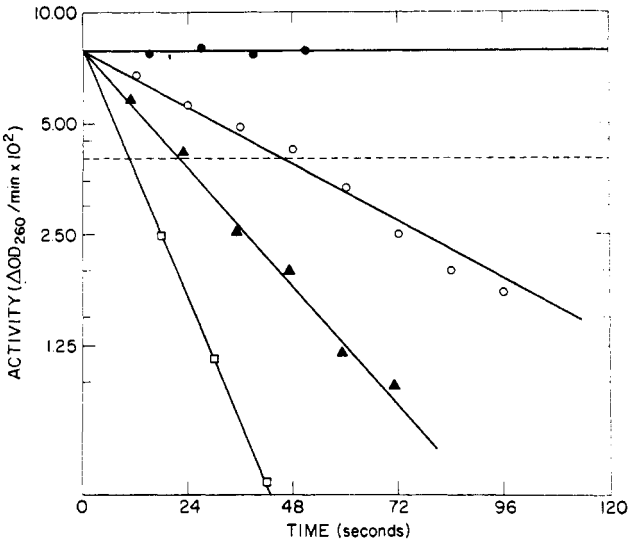

FIGURE 4: Kinetics of nuclease inactivation by anti-$(99–126)_n$. A semilogarithmic plot of activity *vs.* time for assays of 0.05 μg of nuclease in the presence of : (●) 0.00 μg of antibody, (○) 2.85 μg of antibody, (▲) 5.70 μg of antibody, and (□) 11.40 μg of antibody. The dotted line represents one-half of the initial activity.

of the control, it fell to zero well before the time at which it might have been expected to decrease due to limiting substrate. When twice as much anti-$(99–126)_n$ was added initially (lowermost curve), a similar but more rapid curtailment of the nuclease activity was observed. Also illustrated in Figure 2 are the results of repeated additions of equal samples of nuclease to each of the cuvets after all assays had reached plateau values. This was done in order to test the possibility that the rapid fall in slope and small total change in optical density of assays in the presence of antibody might be due to some limitation of available substrate. The absorption in the control cuvet was not appreciably affected by such additions, indicating complete utilization of the substrate. However, both of the antibody containing cuvets demonstrated absorption changes similar to those observed following the first addition of enzyme, indicating the presence of unhydrolyzed substrate susceptible to further nucleolytic digestion.

It seemed likely that these changes in nuclease activity with time reflected the specific interaction of antibody with the enzyme leading to an inactive, soluble antibody–antigen complex. A model for this interaction was formulated as follows

$$\text{Ab} + \text{nuclease} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{Ab–nuclease} \qquad (2)$$

in which Ab refers to anti-$(99–126)_n$ antibodies, k_{on} to the second-order association rate constant for the interaction, and k_{off} to the first-order dissociation rate constant. From the molar concentrations used and the ability of 2.85 μg of antibody to inactivate at least three successive 0.05-μg portions of nuclease, it was clear that the experiments had been performed in large antibody excess and that the concentrations of antibody would not have been expected to change greatly during the inactivations. If the proposed model of interaction were correct, then the inactivation process might be expected to follow kinetics which are first order in nuclease concentration, *i.e.*

$$\frac{d[\text{nuclease}]}{dt} = -k_{on}[\text{Ab}][\text{nuclease}] \qquad (3)$$

$$\frac{d[\text{nuclease}]}{[\text{nuclease}]} = -k_{on}[\text{Ab}]dt \qquad (4)$$

which integrates to

$$\log [\text{nuclease}] = \log [\text{nuclease}]_o - k_{on}[\text{Ab}]t \qquad (5)$$

In order to test this hypothesis, nuclease assays in the presence of varying concentrations of anti-$(99–126)_n$ were performed with the recording spectrophotometer set at an optical density scale tenfold that ordinarily used for nuclease assays (*i.e.*, 0.1 OD_{260} = full scale). The activity curves generated under these conditions (Figure 3) were then analyzed by measuring the differences in OD_{260} (*i.e.*, nuclease activity) between successive points and plotting these differences against the midpoint of each time interval on semilog paper. Data plotted in this fashion could be fitted to straight lines which, on extrapolation to zero time, originated at the same activity as a control sample of nuclease to which no antibody had been added (Figure 4).

Equilibrium Studies. The activities of aliquots from mixtures of anti-$(99–126)_n$ with increasing concentrations of nuclease are plotted in Figure 5. The activities of aliquots of similar

TABLE I: Association Rate Constants According to the Equation $k_{on} = (\ln 2/t_{1/2}[Ab])$.

Ab (μg)	$[Ab]_{sites}$	$t_{1/2}$ (sec)	k_{on} (M^{-1} sec^{-1})[a]
0	0		
2.86	3.81×10^{-8}	46	3.95×10^{5}
2.86	3.81×10^{-8}	45	4.04×10^{5}
5.71	7.67×10^{-8}	22	4.13×10^{5}
5.71	7.62×10^{8}	23	3.96×10^{5}
11.43	1.52×10^{-7}	11	4.15×10^{5}
11.43	1.52×10^{-7}	11	4.15×10^{5}

[a] $(k_{on})_{av} = 4.1 \pm 0.1 \times 10^{5}$ M^{-1} sec^{-1}.

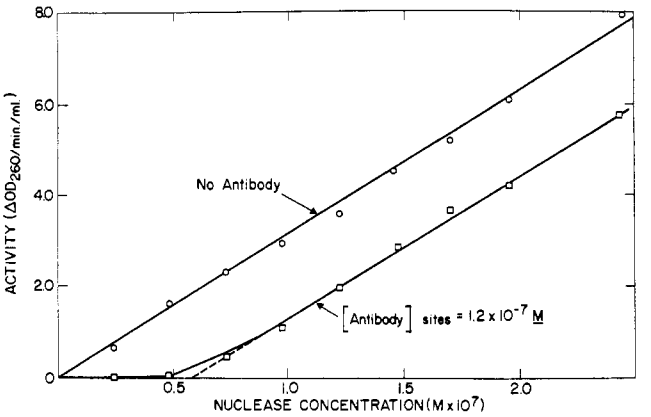

FIGURE 5: Equilibrium study of nuclease inactivation by anti-(99–126)$_n$. Measured enzymatic activities are plotted against total nuclease concentration for each of the equilibrium mixtures. Control mixtures (No Antibody) contained a volume of 0.15 M NaCl equal to the volume of the antibody solution added to each of the experimental mixtures.

concentrations of nuclease in the absence of antibody are included for comparison. The pattern of absorbancy change produced by aliquots of equilibrium mixtures retaining activity showed neither early curtailment nor progressive increase in slope, suggesting that the combination of antibody with nuclease had reached completion during the preincubation and that no appreciable dissociation occurred during the time required for assay. The intercept on the abscissa of the extrapolated linear portion of the curve of activity *vs.* nuclease concentration in the presence of antibody occurred at a nuclease concentration of approximately 6×10^{-8} M (Figure 5), equal to the molarity of the antibody used in these equilibrium studies.

Discussion

The analysis of the interaction of antibodies with naturally occurring macromolecules allows the study of problems which are not readily approached through the use of haptens (Cinader, 1967). For example, the importance of conformation in the antigenic determinants of a protein can be assessed only in polypeptides large enough to generate secondary and tertiary structure. There are, however, two major obstacles to the quantitative study of the interaction of antibodies with naturally occurring macromolecular antigens. First, such antigens often bear multiple antigenic determinants. Since most physical methods available for the study of molecular interactions (*e.g.*, absorption, fluorescence, circular dichroism, or nuclear magnetic resonance spectroscopy) require soluble reactants and products, the precipitation reaction between antibodies and multivalent antigens cannot readily be followed by these means. Second, naturally occurring macromolecular antigens frequently lack chromophores permitting sensitive detection. The use of radioactive labeling with iodine may overcome this problem (Berson and Yalow, 1959; Isagholian and Brown, 1970), but antibodies may bind differently to native antigens than to their iodinated derivatives.

Precipitation has not been a problem in the present study because of the use of fractionated antibodies. Anti-(99–126)$_n$ antibodies are specific for a distinct antigenic determinant of the nuclease molecule and their interaction with nuclease does not lead to precipitation and can be followed spectrophotometrically. Nor has sensitive detection of antigen been a problem in this study since nuclease activity can be measured conveniently by the standard spectrophotometric assay in the range of nuclease concentration of 4×10^{-10} to 6×10^{-9} M. Because the binding of these antibodies to nuclease leads to an inactive complex, this assay provides an extremely sensitive means of monitoring free-antigen concentration during the course of the antibody–antigen interaction.

This sensitivity allows kinetic analysis of the interaction in the time scale of the nuclease assay. The rate of reaction between an antibody and its antigen has usually been considered to be near the theoretical maximum imposed by diffusion limitation on molecules of these sizes (Hammes, 1968). Thus most kinetic studies of the interaction between anti-hapten antibodies and their respective haptens require temperature-jump or stopped-flow techniques with analysis of spectroscopic changes in the microsecond to millisecond range (Hammes, 1968; Pecht *et al.*, 1972). However, from eq 5 for the pseudo-first-order reaction of antibody with nuclease we derive

$$t_{1/2} = \frac{\ln 2}{k_{on}[Ab]} \tag{6}$$

Under pseudo-first-order conditions it is therefore the product of k_{on} and antibody concentration which determines the observed reaction rate. Thus if one works at low antibody concentrations, but in antibody excess, one can obtain measurable half-time values despite very high association rate constants (Sehon, 1963).

Under the assay conditions used in these studies antibody concentration was in great excess and free antibody concentration remained essentially constant during the initial phases of the interaction with nuclease. Thus, using three antibody concentrations in this range ($2–8 \times 10^{-8}$ M), as shown in Figure 4, we have found that the inactivation does indeed follow the predicted first-order kinetics. The values of $t_{1/2}$ and k_{on} calculated from the experimental data of several such experiments are shown in Table I. These results support the proposed model for the antibody–antigen interaction. If one takes into account the expected difference in collision frequency for a molecule the size of nuclease as compared to small haptens, the value we have obtained for k_{on} in our system is in accord with reported values for antibody–antigen binding (Hammes, 1963; Moore, 1962).

The correct interpretation of our equilibrium data is unclear. From the data in Figure 5 it appears that the binding of nuclease by antibody to form an inactive complex is almost

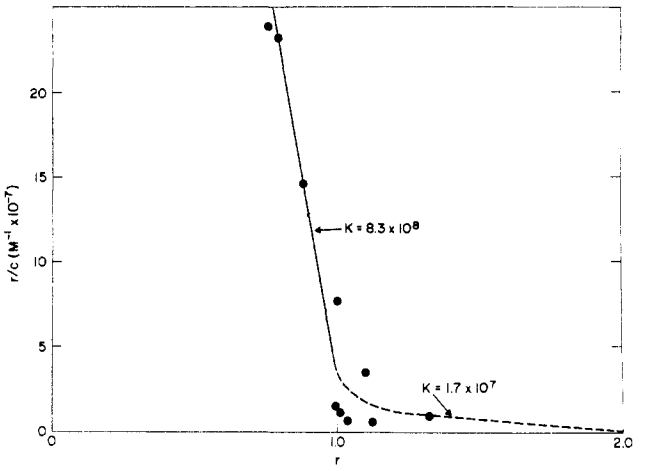

FIGURE 6: Scatchard plot. The binding of nuclease by anti-(99–126)$_n$ is plotted as r/s *vs.* r, values of r being calculated as measured concentrations of bound nuclease per total antibody concentration in equilibrium mixtures. The dotted portion of the curve has been drawn so as to intersect the abscissa at $r = 2$.

complete up to a concentration of nuclease just equal to the antibody concentration. If all binding sites of the antibody molecules were equivalent one might have expected binding up to a concentration twice that of the antibody molarity. The Scatchard plot of data from several equilibrium experiments is shown in Figure 6, values of r being calculated as concentrations of bound nuclease divided by total antibody concentration. Also shown in Figure 6 are the derived values of K_{ass} at two points along this curve (*i.e.*, the negative slopes according to eq 1). The portion of this curve at r greater than one has been drawn so as to intercept the abscissa at $r = 2$, in accordance with known values for γG-immunoglobulins. In the region below $r = 1$ the curve is steep and appears linear, indicating a high and rather uniform K_{ass}. If one extrapolates this portion of the curve to $r/c = 0$, the r intercept is close to 1 rather than to the expected valence of 2, reflecting the same discrepancy that was noted in Figure 5.

One possible explanation for these results is that there are two discrete subpopulations of antibody in anti-(99–126)$_n$, both having the same specificity, but one with an average binding constant of 8.3×10^8 M^{-1} and the other with an average binding constant of 1.7×10^7 M^{-1}. In this case the tight binding of nuclease noted in the data in Figure 5 would be entirely due to the higher affinity antibody and the marked curvature near $r = 1$ in the Scatchard plot would be interpreted as a special case of the nonlinearity often found in such plots and attributed to antibody heterogeneity (Kabat, 1961). This explanation would further require that the two subpopulations account each for just one-half of the total anti-(99–126)$_n$ antibodies. Similar sharply bending curves of r/c *vs.* r have been observed for the binding of γM-immunoglobulins; however, the existence of two discrete populations each accounting for one-half of the total antibody was thought unlikely (Onoue *et al.*, 1968).

Another possible explanation of these results is that although both binding sites of the anti-(99–126)$_n$ antibodies are initially equivalent, the binding of nuclease to one of the sites may impose steric interference to the binding of another nuclease molecule to the second site. Although such interference has not previously been found in binding studies employing haptens, the much larger size of nuclease might make this interaction possible. From the known molecular dimensions of nuclease (Cotton and Hazen, 1971) and the γG molecule (Davies *et al.*, 1971), such steric interference would imply that the binding sites of the two Fab regions of the intact immunoglobulin must face one another. To date, however, there has been no direct crystallographic localization of binding sites by which one might judge this possibility. It is hoped that a choice between these possibilities may be forthcoming from studies now in progress on the binding equilibria of monovalent Fab fragments of anti-(99–126)$_n$.

While the value of $4.1 \pm 0.1 \times 10^5$ M^{-1} sec^{-1}, which we have obtained for k_{on}, is quite precise (Table I), its accuracy is dependent on which of the above interpretations of the equilibrium studies is actually correct. This arises because in the calculation of k_{on} from eq 5 we have computed the concentration of antibody combining sites as twice the total antibody concentration, assuming all antibody binding sites to be equivalent. This assumption should be valid even if the binding of antigen by one site were to affect binding at the second site, since under initial conditions of the kinetic assay in high antibody excess, the fraction of antibody molecules with one site already occupied would be small. However, if there are two subpopulations with different association constants within anti-(99–126)$_n$, then the relevant value for concentration of antibody combining sites should be that of the higher affinity antibodies only. In this case the actual value of k_{on} would be approximately twice that which we have determined, or 8.2×10^5 M^{-1} sec^{-1}. Thus the k_{on} value in Table I is a lower limit.

Since these kinetic studies of antibody-induced inactivation were all carried out in the presence of antibody excess, the equilibrium attained under these conditions would occur at a value of r less than unity. Thus, regardless of the reason for the shape of our Scatchard plot, the relevant K_{ass} for such conditions can be adequately determined from the steep portion of the curve in Figure 6 as 8.3×10^8 M^{-1}. The value of k_{off} may be calculated from the other two experimentally determined values, according to eq 7 and is 4.9×10^{-4} M^{-1}

$$k_{off} = \frac{k_{on}}{K_{ass}} \tag{7}$$

sec^{-1}. From this value one would expect a half-time for the dissociation of the antibody–nuclease complex of

$$t_{1/2} = \frac{\ln 2}{k_{off}} = \frac{0.693}{4.9 \times 10^{-4}} = 23 \text{ min} \tag{8}$$

This must be considered as a minimum value of $t_{1/2}$ since one might expect the k_{off} to be even smaller in greater antibody excess than those from which the association constant was calculated (*i.e.*, if one could determine r/c at lower values of r, the slope of the Scatchard plot would presumably be even steeper). In any case it is obvious from the magnitude of $t_{1/2}$ that dissociation of the antibody–nuclease complex would not proceed appreciably during the time necessary for an enzyme assay.

Implicit in this kinetic analysis has been the assumption that the effects of substrate DNA on the antibody–antigen interaction can be neglected. The justification for this assumption is twofold. First, equilibrium studies in which the preincubation of antibody and nuclease is carried

out in 0.15 M saline or in the DNA substrate solution give identical results. Second, assays of antibody–nuclease equilibrium mixtures in the presence of different substrate concentrations has shown that the inactivation is a noncompetitive form of inhibition, as might be expected from the fact that 99–126 is not directly involved in the enzyme's active site (Arnone *et al.*, 1971; B. M. Dunn and D. H. Sachs, 1972, unpublished data). It would thus appear that anti-$(99–126)_n$ recognizes its antigenic determinant in nuclease equally well in the presence or absence of substrate.

In addition to quantitating the interaction of antibodies with a native protein, the kinetic analysis presented in this paper also provides a rapid and extremely sensitive assay for free antibody. Since the slope of a plot of the logarithm of nuclease activity *vs.* time measures the quantity k_{on}[Ab], one need only add an aliquot of nuclease to a standard assay mixture containing an unknown concentration of antibody and then measure the decrease in nuclease activity in order to quantitate the unknown antibody concentration. The entire assay takes about 2 min and determines antibody concentrations as low as 10^{-8} M. The assay is therefore comparable in sensitivity to assays involving radioactive labeling (Hunter, 1967). We are presently using this assay as a means of quantitating the binding of anti-$(99–126)_n$ antibodies to polypeptide fragments derived from nuclease and will present the results of these studies subsequently.

Added in Proof

Fujio *et al.* (1971) have recently reported the preparation of antibodies to distinct regions of lysozyme by methods similar to ours. They also report the binding constants between these antibodies and lysozyme as determined by equilibrium dialysis using radioactively labeled lysozyme.

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