The Mechanism of Inhibition of Papain by Its Specific Antibodies*

Emmanuel Shapira† and Ruth Arnon

ABSTRACT: The mechanism of inhibition of papain by its specific antibodies has been studied using antibody species of differing inhibitory properties (which had been prepared by selective immunoadsorption). A difference was observed between the various species as regards their relative inhibitory capacity of papain activity on benzoyl-L-arginine ethyl ester (BAEE) and casein, respectively. The more inhibitory antibodies inhibited the activity on BAEE as effectively as that on casein. On the other hand, the antibodies which did not inhibit at all papain activity on BAEE, still inhibited quite effectively the activity on a large substrate. The extent of inhibition does not depend on the sequence in which the reactants, namely antibody and substrate, are added to the enzyme. On the other hand, it depends on the state of aggregation of the antigen–antibody complex. Thus, when the complex is in the form of a precipitate a larger inhibitory effect is observed on papain activity toward casein than when the complexes are soluble. No such effect was observed with BAEE. Kinetic studies of the inhibition indicate that normal immunoglobulin G is a competitor substrate with BAEE for papain activity, its presence resulting in an apparent inhibition of the pure competitive type. The inhibition caused by the specific antibodies is, on the other hand, of the pure noncompetitive type. When the antibodies are used in high concentration a mixed-type inhibition is observed. The K_i values for normal immunoglobulin G and the different species of specific antibodies were calculated.

The interaction between enzymes and their respective antibodies leads generally to a reduction in the enzyme activity, as has been demonstrated for almost every enzyme system studied. Several attempts have been made to elucidate the mechanism of this inhibition; however, it has not been found possible to arrive at a unified concept of the results obtained with a large number of different enzymes (Marrack, 1950; Najjar and Fisher, 1956; Cinader, 1957, 1967; Domardsky, 1961). Regardless of whether a single mechanism can be ascribed to the reduction of the enzymic activity, or whether each enzyme–antibody system represents a separate and unique problem, a number of factors have to be considered when the mechanism of inhibition applicable to a given system is to be evaluated.

The extent of inhibition of an enzyme by its antibodies is related to the size of the substrate, as shown for several enzyme–antienzyme systems (Cinader, 1967). These results led to the conclusion that the inhibition by antibodies is attributable mainly to steric hindrance. This is not necessarily due to antigen–antibody aggregates, as indicated by the finding that monovalent papain-produced antiRNase fragments, which are capable of forming only soluble complexes, inhibited the enzymatic activity of RNase on RNA more efficiently than on citidine 2′,3′-cyclic phosphate (Cinader and Lafferty, 1964). Nevertheless, aggregate formation contributes to steric hindrance as it interferes with the access of substrate to the catalytic site. This aspect has been tested with several enzyme systems (*e.g.*, Arnon and Schechter, 1966; Michaelides *et al.*, 1964; Cinader and Lafferty, 1963, 1964; Alemida *et al.*, 1965). The additional inhibitory effect due to aggregate formation may reflect the relative positioning of the catalytic site and the antibody combining sites on the enzyme.

It has been reported for several enzymes (*e.g.*, Sevag *et al.*, 1954; Samuels, 1961; Michaelides *et al.*, 1964) that the combination with the substrate is accompanied by conformational changes which may prevent subsequent interaction with the antibody. Conversely, once the enzyme has become complexed by the antibody, it may be hindered in reacting with the substrate, or undergoing substrate-induced conformational changes. Hence, when such a mechanism is operative, the extent of inhibition may be expected to differ depending upon the sequence of addition of the reactants.

Finally, the kinetics of the inhibition reaction may help to elucidate the interaction between the enzyme and the antibody. The heterogeneity of antibodies is usually a complicating factor for their use as inhibitors in kinetic studies. Antibodies against different antigenic determinants on the enzyme will vary in their inhibitory capacities, and may compete with each other for the combination with the enzyme. In the special case of proteolytic enzymes, the antibodies or even nonrelated immunoglobulins may serve as potential substrates for the enzyme and, therefore, interfere with the activity of the enzyme on another substrate, by substrate competition. Successful fractionation of antipapain antibodies into populations of different efficiencies of inhibition was expected to be helpful in this respect.

* From the Section of Chemical Immunology, The Weizmann Institute of Science, Rehovoth, Israel. *Received July 18, 1967.*

† On leave of absence from the Pediatric Research Department, Kaplan Hospital, Israel.

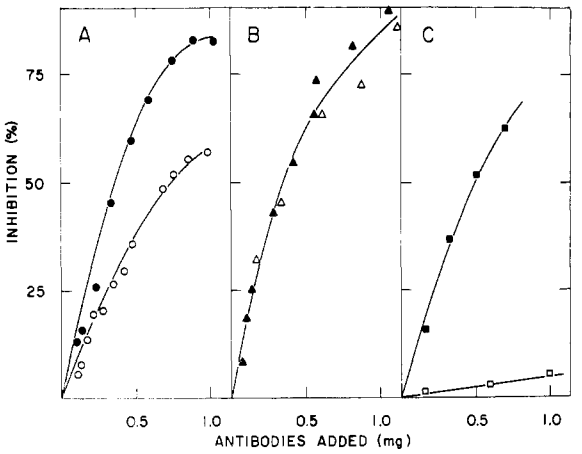

FIGURE 1: Inhibition of papain activity (25 μg of papain) by the various preparations of specific antibodies, as assayed on casein (●, ▲, or ■) and BAEE (○, △, or □). (A) Inhibition by the total antibody population (P). (B) Inhibition by the CP antibodies. (C) Inhibition by the PS antibodies.

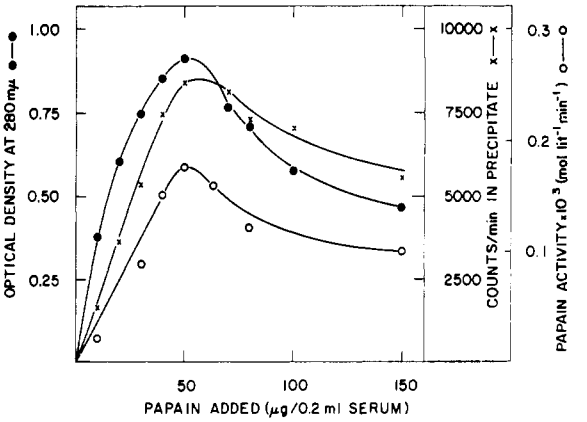

FIGURE 2: Papain activity in immune precipitates. Precipitin experiment was carried out with ^{131}I-labeled papain. Absorbancy at 280 mμ of solution in 0.1 NaOH of precipitates obtained by adding increasing amounts of papain to 0.2 ml of serum (●—●). Counts per minute in the precipitates (○—○), 50 μg of papain correspond to 11000 cpm. Residual papain activity in the immune precipitate, as assayed on BAEE (×—×), 50 μg of the [I^{131}]papain preparation hydrolyzed BAEE in the rate of 0.52×10^{-3} M^{-1} min^{-1}.

In the present publication, we wish to report on studies of the mechanism of inhibition of papain by its specific antibodies, including the effect of each of the factors mentioned above. Only purified antibody preparations, belonging exclusively to the IgG[1] type, were used throughout. The availability of a method for selective separation of the antipapain antibodies into inhibitory and noninhibitory species (Arnon and Shapira, 1967) permitted a better understanding of the role played by the heterogeneity of antibodies in the mechanism of inhibition.

Materials and Methods

Materials. Papain (two-times crystallized, lots 5581, 5623) and chymopapain (lot 5541) were purchased from Worthington Biochemical Co. Banzoyl-L-arginine ethyl ester (BAEE) was obtained from Yeda Co. Casein was purchased from Nutritional Biochemical Co. All other reagents were either reagent grade or the best grade available.

Antibody Purification. The various purified antibody preparations (P, CP, and PS) were isolated with specific papain and chymopapain immunoadsorbents from the IgG fraction of the immune serum, as described in the preceding paper (Arnon and Shapira, 1967).

Enzymatic Assays. Papain activity was assayed in the presence of 0.005 M cysteine and 0.002 M EDTA. With high molecular weight substrate the method was as described in the preceding publication. The activity of papain on low molecular weight substrate was assayed by following the hydrolysis of BAEE at pH 6.5 and 37°, using an autotitrator (Radiometer, Copenhagen Model TTT 1, with a glass–calomel combined electrode No. GK 2021c or GK 2641 C). The pH 6.5 was chosen for the reaction since, according to Sluyterman (1964), no product inhibition occurs in this range.

Inhibition assays of enzymatic activity, quantitative precipitin tests, and other immunological procedures, as well as physical methods, were all performed as described in the preceding paper.

The K_m value and the K_i values for the different antibody species were calculated from the slopes of the respective Lineweaver and Burk's plots according to Dixon and Webb (1964).

Results

Correlation between Size of Substrate and Extent of Inhibition. In the preceding paper we described the selective fractionation of antipapain antibodies into a species of high inhibitory capacity (denoted "CP"), and a noninhibitory species (denoted "PS"). As these results were obtained only with a low molecular weight substrate (BAEE), the study was extended to include a high molecular weight substrate, casein. The extent of inhibition in each case was determined by following the residual enzymatic activity after 1-hr incubation of the enzyme with the particular antibody sample.

Figure 1 presents the extent of inhibition affected by each of the antibody species on the two substrates. As shown, the extent of inhibition by the total antibody

[1] BAEE, benzoyl-L-arginine ethyl ester; IgG, rabbit immunoglobulin G fraction; CP, antibodies purified from antipapain IgG fraction with chymopapain immunoadsorbent; PS, antibodies adsorbed with papain immunoadsorbent following exhaustive adsorption with chymopapain immunoadsorbent; P, total antibody population purified from the antipapain IgG fraction by papain immunoadsorbent.

population (Figure 1A) was found to be different for the two above-mentioned substrates, whereas the more efficient inhibitor (CP) had a very similar effect on both BAEE and casein (Figure 1B). On the other hand, the species which had almost no inhibitory capacity as regards papain activity on BAEE (PS), was an effective inhibitor when assayed on casein (Figure 1C).

The Role of Aggregate Formation in Inhibition. The enzymatic activity of the papain–antipapain mixture persists in the precipitates which are formed during the antigen–antibody interaction. This was demonstrated by the following experiment. A precipitin reaction was carried out in duplicate, using ^{131}I-labeled papain, and the amount of papain in the washed precipitates was quantitated by monitoring the radioactivity in each of the samples. One set of precipitates was used for the determination of the protein content, while in the other sets of tubes the precipitates were suspended in 0.08 M BAEE solution (containing activating agents) for determination of the residual papain activity. As seen in Figure 2, in the entire range of the precipitin curve the precipitates possess enzymatic activity. Moreover, the relation between residual activity and total amount of enzyme present in the precipitate is maintained constant throughout the precipitin curve.

In order to determine whether the state of aggregation has any effect on the extent of inhibition, an experiment was performed in which the activity in the precipitates was compared to that of soluble complexes formed from these precipitates. Use was made here of the fact that, since the antigen is papain, the addition of papain-activating agents to the reactants in the precipitin reaction will bring about the formation of soluble complexes of papain with Fab fragments of the antibody (Arnon, 1965). The experiment was, therefore, performed in duplicate sets. In the first set, the antibody was incubated with the enzyme, and the activating agents were added just prior to the activity assay. The interaction of the enzyme with the antibody was indicated by the appearance of a heavy immune precipitate, which did not dissolve during the enzymatic assay. In the duplicate set, the activating agents were added simultaneously with the antibody, not allowing the formation of precipitates during the incubation. The enzymatic activity in the precipitates and in the soluble complexes was determined using both BAEE and casein, as substrates, and the results are summarized in Table I. When BAEE was used as the substrate, no difference could be detected between the extent of inhibition in the two sets. On the other hand, when the substrate was a large molecule, the aggregation to form a precipitate increased the extent of inhibition. It is interesting to note that when the papain is in the form of soluble complexes with the antibody fragments, the same extent of inhibition is observed in both assays, whether casein or BAEE are used as substrates.

Substrate Protection in Inhibition Studies. To determine whether the interaction of the enzyme with its substrate has any protecting effect against the inhibition by the antibodies, the sequence of addition of the reactants was varied. Thus in one case, the enzyme was preincubated with different amounts of the antibody, in the presence of activating agents; the substrate (0.1 M BAEE) was added after 1 hr, and the rate of BAEE hydrolysis was recorded. In the second case, the activated enzyme was first allowed to react on BAEE; the rate of the reaction was recorded with the pH-Stat for 6–8 min, following which the antibodies were added. As shown in Figure 3A, the decrease, for a given antibody concentration, in the rate of hydrolysis of BAEE was observed immediately upon addition of the antibodies (curve I). The extent of inhibition, as indicated by the slope of the curve, was exactly the same as that attained by preincubation of the papain with the same amount of antibodies (curve II). Figure 3B demonstrates the calculated extent of inhibition as a function of antibody concentration. As shown, the same inhibitory effect is brought about regardless of the sequence of addition of the reactants.

TABLE I: Role of Aggregation in Inhibition.

Antibody Added (mg)[a]	Extent of Inhibition (%)			
	Assayed with BAEE		Assayed with Casein	
	Ag–Ab Ppt	Soluble Complex	Ag–Ab Ppt	Soluble Complex
0.16	12	12	24	11
0.31	25	25	40	23
0.47	36	36	55	37
0.63	45	45	74	42

[a] All experiments were carried out with 25 μg of papain.

Kinetics of Inhibition. Since the antibodies consist of immunoglobulin molecules, which are capable of being hydrolyzed by papain, it had to be established first whether substrate competition exists between normal immunoglobulins and BAEE. For this purpose the hydrolysis of BAEE by papain was followed in the presence of increasing concentrations of normal globulins. The results, plotted according to Lineweaver and Burk, are given in Figure 4. As shown, normal rabbit immunoglobulin G indeed competes with BAEE for the papain activity, an effect which is manifested as an apparent inhibition. This inhibition is of the strictly competitive type, with an average apparent K_i value of 5.15×10^{-5} M. (The K_m value obtained for papain under the same experimental conditions was 0.023 M.)

The type of inhibition caused by the specific antibodies is completely different from that caused by normal immunoglobulins. Figure 5 shows the double-reciprocal plots obtained by the method of Lineweaver

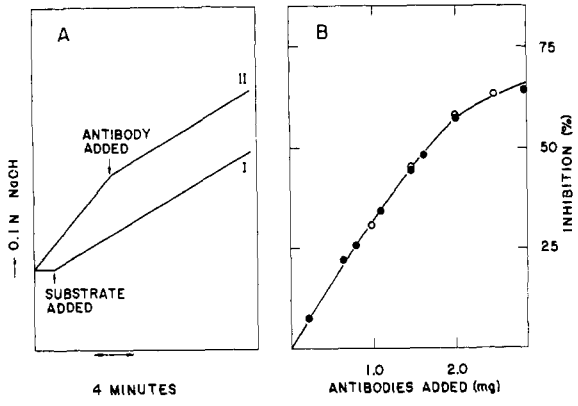

FIGURE 3: Effect of substrate on papain (50 μg) inhibition by purified antibodies (P). (A) Progress curves of BAEE (0.1 M) hydrolysis. In curve I papain was incubated with the 1.5 mg of antibody for 1 hr, and the substrate was added at the point indicated by the arrow. In curve II the enzyme was first allowed to react on BAEE and the same amount of antibody was added at the point indicated by the arrow. (B) Calculated extent of inhibition as a function of antibody concentration. (●—●) Enzyme was preincubated with the antibody for 1 hr prior to addition of substrate. (O—O) Antibodies added to reacting enzyme, 6–8 min after initiation of hydrolysis.

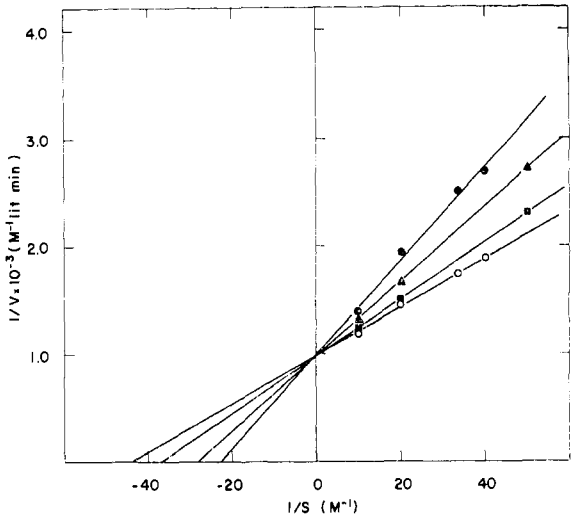

FIGURE 4: Effect of normal IgG on papain hydrolysis of BAEE. The papain concentration in all experiment 12.5 μg/ml. Concentrations of IgG are the following: (O—O) no IgG added, K_m value 2.3×10^{-2} M; (■—■) 1.25 mg/ml, K_i value 4.9×10^{-5} M; (▲—▲) 5 mg/ml, K_i value 5.2×10^{-5}M; and (●—●) 7 mg/ml, K_i value 5.3×10^{-5} M.

and Burk for the specific antibodies. It is clear that in contrast to the normal globulins, the inhibition both by the total antibody population and by the antibodies of the CP species (the more effective inhibitors) was of the noncompetitive type. The average K_i value obtained for the total antibody population was 10.3×10^{-6} M, while the K_i for the CP antibodies was 4.6×10^{-6} M. The interaction with the antibodies (in a concentration range up to 1 mg/ml) did not cause any change in the K_m value of papain, which remained 0.022–0.024 M. The only effect observed was a decrease in the V_{max}.

The inhibition by the normal globulins of the action of papain on BAEE is an order of magnitude less efficient than that caused by antipapain antibodies, and indeed it is apparent only at concentrations higher than 1.25 mg/ml (per 12.5 μg/ml of papain). On the other hand, the specific antibodies, under similar conditions, approach maximum inhibition at a concentration of 1 mg/ml (see Figure 1). When the effect of the specific antibodies is tested at higher concentrations, the resultant inhibition ceases to be purely noncompetitive and goes over into the mixed type (competitive and noncompetitive), as seen in Figure 6. This figure gives the Lineweaver–Burk plot, at different concentrations, for both the total antibody population and the selectively fractionated inhibitory antibodies. At lower concentrations, both antibody preparations are shown to yield a plot typical for noncompetitive inhibition (these represent the same data as those given in Figure 5, but on a different scale). At higher concentrations, the mixed-type inhibition is observed. It is pertinent to remark that at a concentration of 1 mg/ml the two different antibody preparations exhibit different type of inhibition, whereas the inhibition by the total antibody population is pure noncompetitive, the CP antibodies cause mixed-type inhibition. The possible explanation of this phenomenon will be dealt with in the Discussion.

Discussion

The existence of a correlation between the extent of inhibition and the size of the substrate has been reported for several enzyme–antienzyme systems (Branster and Cinader, 1961; Fazekas de St. Groth, 1963; Lepow, 1963; Arnon and Schechter, 1966). However, upon introducing an additional parameter, namely, the prior selective separation of specific antipapain antibodies into fractions of differing inhibitory capacity, we found that the correlation between the inhibition and the size of the substrate is entirely dependent on the specific type of antibody involved, and therefore varies for the three species under discussion (Figure 2). The fact that the most inhibitory antibodies (CP), *i.e.*, those isolated on insolubilized chymopapain, inhibited the papain activity to the same extent regardless of the size of the substrate suggests that this type of antibodies interacts with those antigenic determinants on the papain which are very close to the catalytic site. This interaction will, therefore, interfere with the enzyme activity even on a low molecular weight substrate. On the other hand, the antibodies of the PS species, *i.e.*, those isolated on

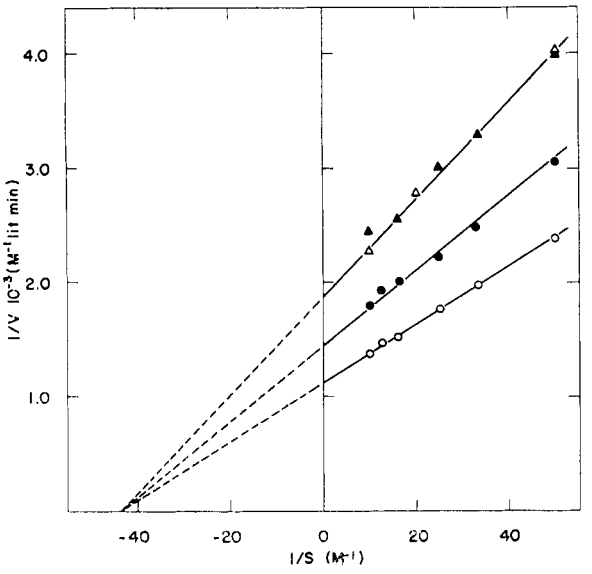

FIGURE 5: Typical noncompetitive inhibition of papain hydrolysis of BAEE by specific antibodies. Papain concentration in all experiments was 12.5 μg/ml. (O—O) No antibody added, K_m value 2.3 $\times$ 10^{-2} M. (●—●) Antibodies of P species, 0.5 mg/ml, K_i value 10.7 $\times$ 10^{-6} M. (▲—▲) Antibodies of the P species (1 mg/ml), K_i value 9.9 $\times$ 10^{-6} M. (△—△) Antibodies of the CP species (0.5 mg/ml), K_i value 4.6 $\times$ 10^{-6} M.

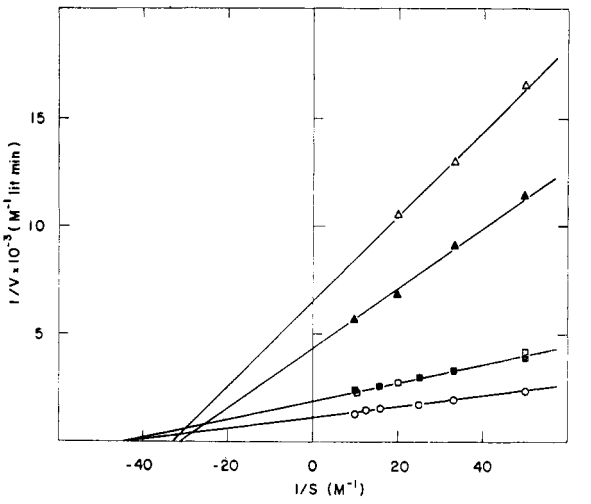

FIGURE 6: Analysis of the type of inhibition by different concentration ranges of specific antibodies. Papain concentration in all experiments was 12.5 μg/ml. (O—O) No antibodies added. (■—■ and □—□) Noncompetitive inhibition by the P antibodies (1 mg/ml) and CP antibodies (0.5 mg/ml), respectively. (▲—▲ and △—△) Mixed-type inhibition by the P antibodies (2 mg/ml) and CP antibodies (1 mg/ml), respectively.

insolubilized papain after the prior removal of CP antibodies, reacted with antigenic determinants that are probably remote from the catalytic site. In consequence of such interaction the access of a large molecular weight substrate would be barred, and hence the papain activity on casein would be inhibited to a fairly large extent, as indeed was the case. On the other hand, it would not interfere at all, with the catalytic action of the enzyme on a small molecular weight substrate. The inhibition of papain by the CP antibodies as regards both substrates is extremely efficient. It can be calculated from Figure 1B that 50% inhibition is obtained when the molar ratio of antibody to the enzyme is 2:1 (0.35 mg of antibody/25 μg of papain).

The degree of inhibition of the activity of the enzyme, on both substrates, by the total population of the specific antibodies, represents approximately the average of the inhibition values obtained with each of the two antibody fractions (CP and PS). Thus, using the total antibody population, the efficiency of inhibition as regards the activity on casein did not decrease significantly as compared to the case where the CP antibodies were used. On the other hand, using BAEE as substrate, a molar ratio 4:1 of antibody to enzyme was required to yield 50% inhibition (Figure 1A). It is reasonable to assume, therefore, that the inhibition in such a heterogeneous system depends on the composition of the antibody population and on the relative proportions in which the different species are present.

The role of aggregation in the inhibitory effect of the antibodies is also dependent on the size of the substrate. In the study reported here, the formation of an immune precipitable was prevented by performing the antigen–antibody interaction in the presence of papain-activating agents. As has been shown previously (Arnon, 1965), such treatment leads to the formation of soluble complexes of papain with the Fab fragments of the antibodies. Hence, the disaggregation is in this case accompanied by the reduction of the size of the inhibitor molecules. Nevertheless, this did not bring about any change in the inhibitory effect on the action of papain on the low molecular weight substrate (Table I). These findings are consistent with those reported for carbamyl phosphate synthetase (Marshall and Cohen, 1961), according to which no difference in the inhibitory capacity was observed between purified antibody or its univalent fragments, regarding the synthesis of citrulline. On the other hand, the inhibition of the hydrolysis of casein by the antigen–antibody precipitate was greater than that shown by the soluble complexes, indicating that the aggregation interferes with the access of the large substrate to the catalytic site. This interference may be explained by the direct effect of the more compact structure of which a precipitate consists, namely by assuming that the aggregation prevents the diffusion of the large substrate into the three-dimensional lattice of the immune precipitate. Alternatively it may be explained by the fact that the size of the inhibitor in the soluble complexes, namely the Fab fragment, is smaller than that of the whole antibody molecule, and therefore, since the inhibition is caused by steric hindrance, differences

are observed between substrates of different sizes. A similar phenomenon, namely a difference in inhibition efficiency of Fab fragments regarding substrates of differing size, was observed with ribonuclease (Cinader and Lafferty, 1964).

The extent of inhibition by the antibodies does not seem to depend on the sequence of the addition of substrate and antibodies to the papain. Thus it appears that the catalytic action of papain on BAEE is not accompanied by conformational changes to an extent sufficient to prevent subsequent binding of the antibody. These findings are in agreement with those reported for some enzyme systems (Mansour *et al.*, 1954; Nisselbaum and Bodansky, 1959), but in contrast to the results obtained with many other enzymes (*e.g.*, Smith *et al.*, 1952; Kistner, 1959).

In connection with the kinetic studies, it should be stressed that such experiments are meaningful only if purified antibodies are used, since only such preparations are completely free from any proteolytic or nonimmunologic antiproteolytic activity (Arnon and Shapira, 1967). Moreover, the fact that normal immunoglobulin molecules serve as a competitive substrate for the papain activity with BAEE emphasizes further the absolute requirement for immunospecifically purified antibodies. In suitable concentration, the effect of the antibodies is of the strictly noncompetitive type, *i.e.*, the inhibition is caused only by the formation of antigen–antibody complexes. On the other hand, when antibody is in excess, *i.e.*, when more antibodies are present than are needed for maximum inhibition, the "extra" antibodies apparently behave like nonspecific globulins and compete with BAEE for the residual activity of the papain. The type of inhibition observed in this region is, therefore, "mixed" competitive and noncompetitive. This phenomenon is observed for the CP antibodies at lower concentration than for the total antibody population (P), since the former are more effective inhibitors and, therefore, a smaller concentration is required to bring about maximal inhibition of the papain. This adds further support for the assumption postulated previously (Arnon and Shapira, 1967) that the CP antibodies can react with only part of the antigenic determinants, whereas the P antibodies react with a larger number of antigenic determinants, for the saturation of which larger amounts of the antibodies are required.

In conclusion, in similarity to other enzyme–anti-enzyme systems, the inhibition of papain by its antibodies involves steric hindrance to the access to the catalytic site. But, following selective fractionation of the antibodies a species is obtained, the inhibition by which, though of the noncompetitive type, is brought about mainly by interactions with loci that are in the immediate contiguity of the catalytic site.

Acknowledgments

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