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ANTIGEN-ANTIBODY INTERACTIONS AND THE ANOMALOUS KINETICS OF ARYLSULFATASE A

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Summary

Antibodies against homogeneous rabbit liver arylsulfatase A (aryl-sulfatase sulfohydrolase, EC 3.1.6.1) were produced in a goat and the effects of these antibodies on the kinetic parameters of the enzyme have been studied. The results indicate that the binding of antibody to the enzyme does not alter the enzyme active site, since K_m and K_i values are unaffected. However, a small reduction in the enzyme activity was observed as the result of a reduction of V in the enzyme-antibody complex. The binding of antibodies led to a change in the pH-rate profile, giving one broad pH optimum shifted toward higher pH value. The enzyme-antibody complex still showed the characteristic aryl-sulfatase A anomalous kinetics at pH 5.5, but the inactivation was significantly slower than for the native enzyme. As calculated from quantitative immuno-precipitation data, the native enzyme bound 5—7 molecules of IgG. The number of IgG molecules which bound to the turnover-modified enzyme was reduced to 3—4. The loss of antigenic determinants from the turnover-modified enzyme indicates that significant conformational changes occur during the turnover-induced modification, or that a covalent modification of residues present at the antigenic sites has occurred, or both.

Introduction

The effects of antibodies on enzymes and the nature of these effects have been of considerable interest during the last two decades. Some antibodies

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inhibit the corresponding enzymatic reactions [1—3], while others are without effect or may even enhance the enzymic reactions [4—6]. Quantitative studies of the interaction of antibodies with enzyme antigens may be used in favorable cases to obtain information about structure-function correlations, evolutionary relationships and about conformational changes of enzyme molecules [7—9]. Arylsulfatase A (aryl-sulfate sulfohydrolase, EC 3.1.6.1) has been isolated in a pure form from several different sources [10—13]. Although many physicochemical properties and the catalysis of hydrolysis of synthetic substrates have been investigated [13—16], the immunochemical properties of these enzymes have not been well defined. The presence of inactive mutant forms of arylsulfatase A in the genetic disease metachromatic leukodystrophy [5,17] also emphasizes the need for more detailed studies of the immunochemical properties of the normal enzyme isolated from a convenient source.

The present report describes the effect of antigen-antibody complex formation on the kinetic properties of rabbit liver arylsulfatase A. This enzyme shows the so-called anomalous kinetic behavior similar to that exhibited by arylsulfatase A enzymes of other vertebrates [18—20]. It is slowly modified during the hydrolysis of substrates such as nitrocatechol sulfate and this results in the progressive inactivation of the enzyme. The modified enzyme can apparently be reactivated in the presence (simultaneously) of substrate and sulfate ion. The relative amount of native and modified enzyme in such reaction mixtures is affected by sulfate and other ions such as phosphate and pyrophosphate but only in the presence of substrate. The structural basis of the modification is unknown. The observed temperature dependence of the conversion of modified to native enzyme suggested that the rate-determining step of this process was associated with a small net conformational change [11]. Because prior or subsequent conformational alterations in the protein molecules might lead to changes in antigenic reactivity, we have now conducted immunochemical experiments in order to compare the antigenicity of the native form with that of the inactive, turnover-modified form of the enzyme.

Experimental procedures

Many of the kinetic experiments were done with partially purified enzyme [11,21] having a specific activity of 120 units/mg. For quantitative immunoprecipitation experiments, homogeneous enzyme with a specific activity of 350 units/mg was used. Turnover-modified enzyme was kindly supplied by Dr. A. Waheed.

Enzyme assay

Usually the reaction mixture was 4 mM nitrocatechol sulfate (Sigma Chemical Co., St. Louis, MO) in 0.5 M acetic acid/sodium acetate buffer, pH 5.5, $I = 0.48$ M. An appropriate volume (up to 30 μl) of the enzyme sample or immune precipitate suspended in saline was added to 1.8 ml of reaction mixture. The reaction was quenched after 3 min incubation at 37° C by addition of 1 ml of 2 M NaOH. Free nitrocatechol was determined spectrophotometrically at 515 nm. A molar extinction coefficient $\epsilon = 12\,600\ M^{-1} \cdot cm^{-1}$ was used for calculations. The activity of the turnover-modified enzyme was estimated in

this way after 15 min incubation in a reaction mixture containing additionally 3 mM sulfate.

Antibodies against rabbit liver arylsulfatase A were raised in a goat. 2 mg homogeneous enzyme was used in the immunization procedure (carried out by Antibodies, Inc., Davis, CA). 1 mg enzyme with complete Freund's adjuvant was used for first injection. After 14 days the goat was challenged with the next 1 mg portion of enzyme and complete Freund's adjuvant. Antiserum obtained at 28 days following the first injection of the antigen was used for the isolation of the γ-globulin fraction.

The crude γ-globulin fraction containing precipitable antibodies against the enzyme was isolated from goat antiserum by precipitation three times with $(NH_4)_2SO_4$ to a final salt concentration of 1 M [122]. For quantitative immunoprecipitation experiments this fraction was further purified by chromatography using Sephadex G-200 (Pharmacia, Uppsala) equilibrated with 0.1 M Trsi-HCl, pH 7.5, containing 0.1 M NaCl. This preparation contained no high molecular weight antibodies (IgM) but immunoelectrophoresis showed traces of β-globulins. The IgG concentration was determined spectrophotometrically at 280 nm using an extinction coefficient $E^{1\%}_{cm} = 14$ [23]. Double immunodiffusion tests and immunoelectrophoresis were done using published techniques [24,25]. Normal goat IgG and rabbit antiserum against goat serum were obtained from Miles Laboratories.

Labeling procedure

The Chloramine T-method [26] was used to label goat IgG with ^{125}I. 7 mg goat IgG was isolated from antiserum and dissolved in 0.5 ml of 0.05 M phosphate buffer (pH 7.0) and this solution was mixed with 2 ml of $K^{125}I$ (Amersham-Searle) diluted in 0.2 ml phosphate buffer. 0.2 ml 1 mg/ml Chloramine-T solution was added. After 5 min an equal volume 1 mg/ml $NaHSO_3$ was added. followed by 0.2 ml 3 mg/ml KI. The mixture was quickly applied on a previously calibrated G-25 column (Pharmacia, Uppsala). The labeled IgG fraction was eluted and then exhaustively dialysed against pH 7.5 sodium phosphate/NaCl solution and stored at −20° C. Before use an appropriate volume was diluted using unlabeled goat IgG solution and dialysed against the reaction buffer.

Immunoprecipitation

Quantitative studies of the reaction of arylsulfatase A with antibody were patterned after Ref. 27. To a constant amount of enzyme increasing amounts of IgG were added. The samples were incubated in 0.01 M imidazole hydrochloride/NaCl (pH 7.5) for 2 h at 37° C and then kept for 24 h at 4° C. The mixtures were centrifuged at 4° C and the unprecipitated antigen was assayed by measuring the enzyme activity in the supernatant. The immune precipitates were washed three times using cold 1% NaCl solution, dissolved in 0.2 ml of 0.1 M NaOH and the protein was estimated by the method of Lowry et al. [28] using a calibration curve established for normal goat IgG. The amount of antigen in the precipitate was calculated by subtracting the amount of antigen in the supernatant from that of total antigen added. The amount of antibody in the precipitate was obtained by subtraction of the value due to antigen from

the total amount of protein in the precipitate. The molar ratio of IgG to antigen was calculated assuming molecular weights of 140 000 for arylsulfatase A monomer [21] and 160 000 for goat IgG [29]. A similar procedure was used for radioimmunoprecipitation experiments. The radioactivity in the washed immunoprecipitates was determined after solubilization in 0.2 ml 1 M NaOH plus 10 ml Biofluor (New England Nuclear) using a Packard Tri-Carb Model 3320 liquid scintillation counter. The number of IgG molecules attached to the enzyme molecule was calculated from simultaneous determinations of the radioactivity of standard solutions containing known amounts of labeled goat IgG.

Results

When arylsulfatase A was reacted with IgG isolated from goat antiserum, approx. 50% of the enzyme was precipitated within 15 min (Fig. 1). The disappearance of enzymic activity from the supernatant fraction was a consequence of the complete binding of the enzyme in an insoluble complex with antibodies. This complex still had catalytic activity (Fig. 2), but the total activity of the supernatant plus that in the pellet never reached the activity of the controls. The binding of antibodies to the enzyme slightly inhibited the enzyme activity. With increasing amounts of added IgG, about 30% inhibition was eventually observed. Even with an excess of added antibodies the percentage of inhibition did not increase above this value. K_m values for the enzyme in the complex are very close to the value found for the native enzyme (Table I). Furthermore, the enzyme in the antibody complex is also inhibitied competitively by phosphate and by sulfate ions and the K_i values estimated for antibody-bound enzyme do not differ significantly from K_i values determined for

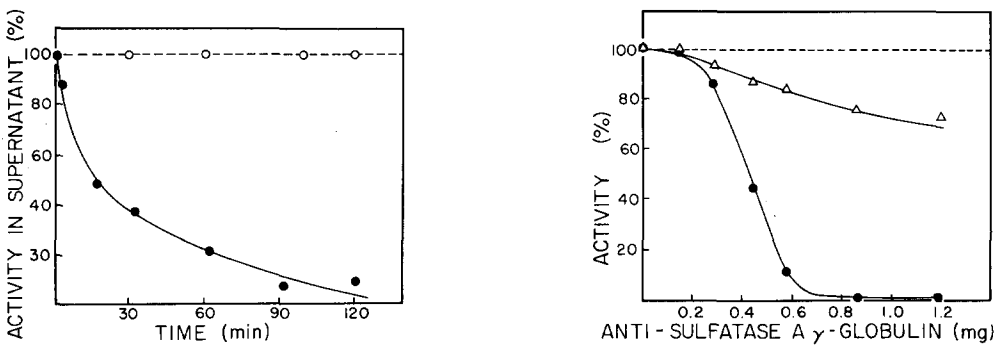

Fig. 1. The rate of formation of insoluble enzyme-antibody complex. The enzyme was incubated at 37°C in 0.01 M imidazole HCl buffer in saline pH 7.5 with an excess of IgG isolated from antiserum. At different time intervals the samples were centrifuged and activity in the supernatant was determined. Normal goat IgG was added to the control samples, which contained the same amount of enzyme protein. ●, activity in the supernatant; ○, activity in the control sample.

Fig. 2. The inhibition of arylsulfatase A activity by addition of anti-sulfatase A antibody. Increasing amount of γ-globulin fraction were added to 1.8 μg enzyme. The samples were incubated for 2 h at 37°C in 0.01 M imidazole hydrochloride/NaCl (pH 7.5) and then kept overnight at 4°C. After centrifugation the activity of the supernatant solution and the pellet were independently estimated. ●, activity in supernatants; △, total activity in supernatant and pellet; - - - - -, activity of control samples.

TABLE I

KINETIC CONSTANTS FOR THE ENZYME-ANTIBODY COMPLEX AND NATIVE RABBIT LIVER ARYLSULFATASE A

Kinetic parameters were determined at 37°C in 0.2 M acetate buffer (pH 5.5) or in 0.1 M acetate buffer (pH 4.5) (values in parentheses). K_m and V values were calculated from Lineweaver-Burk reciprocal plots and K_i values from a Dixon plot of $1/v$ versus [I] in the presence of different concentrations of substrate. K_i values for the native enzyme are from Ref. 11.

	K_m (mM)	V (μmol nitrocatechol · $min^{-1} \cdot mg^{-1}$)	K_i phosphate (mM)	K_i sulfate (mM)
Enzyme-antibody complex	0.87 (0.87)	300 (222)	0.042	2.8
Native enzyme	0.87 (0.87)	452 (296)	0.034	2.9

native enzyme (Table I). V is the kinetic parameter which is significantly changed in the antibody complex. These data indicate that the inhibition of the activity of arylsulfatase in the antibody complex shows a pure noncompetitive character.

The effect of varying pH on activity of the enzyme in the antibody complex is shown in Fig. 3. Although the pH profile for the native enzyme exhibits two regions of optimal activity [16], the pH curve for the antibody complex shows one rather broad pH optimum without the inflection which seems characteristic of the native enzyme isolated from mammalian tissues [16,18].

The enzyme in the immunoprecipitate displays a time-dependent kinetic behavior similar to that of the native enzyme. Fig. 4 presents a comparison of the progress curves at different pH values for the hydrolysis of nitrocatechol sulfate by the complex and by the native enzyme. The time-dependent loss of activity due to the progessive inactivation of the enzyme during reaction at pH 5.5 was observed for the native enzyme and for the complex. The slower inactivation of enzyme in the complex during reaction may due to the lower initial velocity of the enzyme which has been inhibited by antibodies. The addition of sulfate ions to the reaction mixture after 20 min increased the activity of the enzyme in the complex as well as of the free enzyme at the later stages of reaction. At pH 4.5 the enzyme in the complex and the enzyme alone do not appear to exhibit the turnover-induced inactivation. After correction for product inhibition, or in the presence of high substrate concentrations, the substate was cleaved at an almost constant rate [21]. Moreover, the addition of sulfate did not change the profiles of the hydrolysis curves (Fig. 4).

To describe the anomalous kinetics of the enzyme in the complex at 5.5, the mathematical correlations derived by Stinshoff [20] were applied (but see also Ref. 30). The initial velocity of the reaction catalyzed by the enzyme-antibody complex was related to the substrate concentration but was always lower than the initial velocity of the control. U, the maximum turnover achievable, was independent of substrate concentration (Table II).

If the inactivation of the enzyme during reaction with nitrocayechol sulfate is accompanied by a conformation change or a covalent modification of the protein, then it might be possible to detect differences in the antigenicity of the native versus the turnover-modified enzyme. Double immunodiffusion tests in

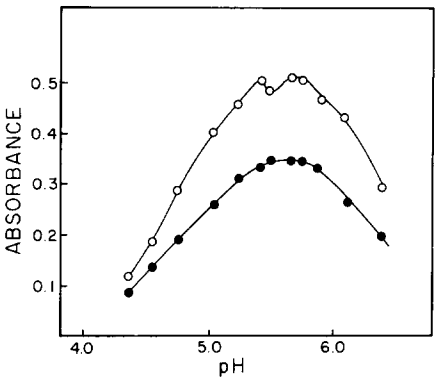

Fig. 3. The effect of pH on the activity of the enzyme-antibody complex. The enzyme-antibody complex suspended in saline was incubated for 3 min at 37° C with 4 mM substrate in 0.5 M acetate buffer at varying pH. •, activity of pellet; ○, activity of control.

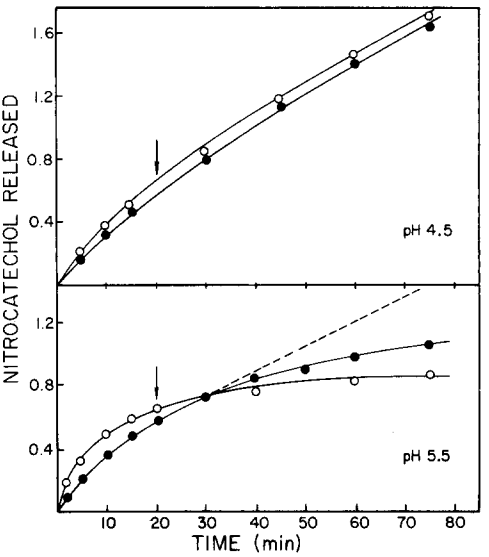

Fig. 4. Progress curves for hydrolysis of nitrocatechol sulfate. The enzyme-antibody pellet suspended in saline was added to 2 ml 8 mM nitrocatechol sulfate in acetate buffer at 37° C. At appropriate time intervals 0.1-ml aliquots were withdrawn from the reaction mixture and added to 1 ml 1 M NaOH. The liberated nitrocatechol was determined spectrophotometrically. Control samples contained the same amount of enzyme protein as the enzyme-antibody pellet together with the appropriate amount of goat IgG from nonimmunized animals. •, activity of enzyme-antibody complex; - - - - - -, activity of enzyme-antibody complex after addition of SO_4^{-2} to final concentration of 3 mM; ○, activity of control. Arrows indicate when SO_4^{-2} (as Na_2SO_4) was added to the reaction mixture.

fact show a reduced immunochemical reactivity of the turnover-modified enzyme with no visible spurs (Fig. 5). A quantiative comparison is possible. If the maximal number of IgG molecules which bind to a molecule of native enzyme (Fig. 6) is compared to the number of IgG molecules which bind to

TABLE II

EFFECT OF SUBSTRATE CONCENTRATION ON THE HYDROLYSIS OF NITROCATECHOL SULFATE CATALYZED BY RABBIT LIVER ARYLSULFATASE A AND ITS ANTIBODY COMPLEX

The values of U (the maximum turnover) and $t_{1/2}$ [20] are given for comparative purposes; for a further discussion of the significance and accuracy of these parameters see Ref. 30.

Native enzyme				Enzyme-antibody complex			
[substrate] (mM)	U (μmol · μg^{-1})	V_0 (μmol · min^{-1} · μg^{-1})	$t_{1/2}$ (min)	[substrate] (mM)	U (μmol · μg^{-1})	V_0 (μmol · min^{-1} · μg^{-1})	$t_{1/2}$ (min)
1	14.7	0.23	26	1	9.8	0.07	60
2	14.7	0.34	18	2	9.8	0.12	35
4	14.7	0.50	12	4	9.8	0.18	23
8	14.7	0.56	11	8	9.8	0.22	19

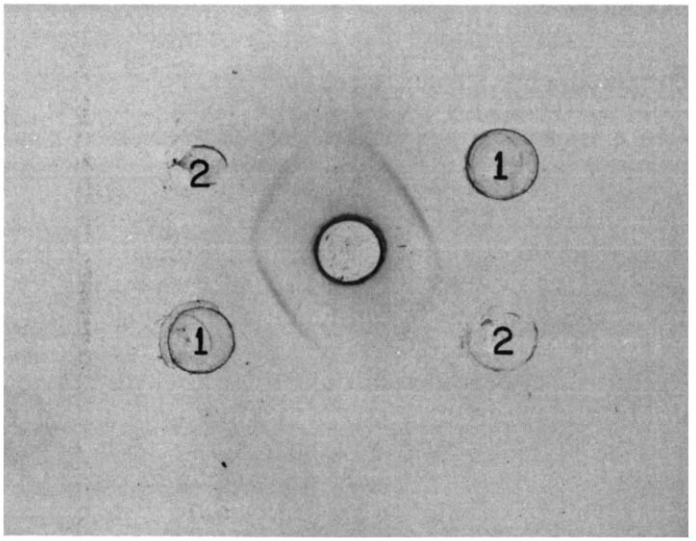

Fig. 5. Immunodiffusion of native and turnover-modified arylsulfatase A. The central well contained goat antiserum against native enzyme; well 1, native enzyme; well 2, turnover-modified enzyme. The protein concentration of native and modified enzyme were the same.

turnover-modified enzyme (Fig. 7), it is found that there is a decrease in the effective concentration of antigenic determinants on the modified enzyme compared to the native enzyme. Radioimmunoprecipitation yielded slightly lower absolute values than those obtained by an estimation of the protein in

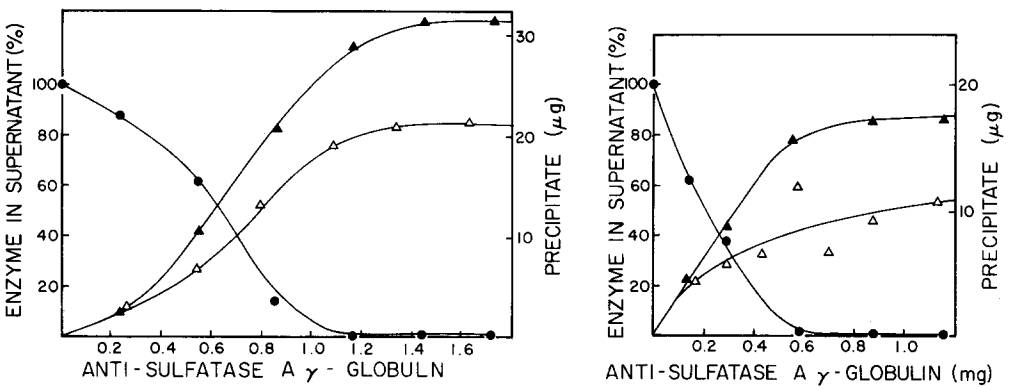

Fig. 6. Precipitation curve for native enzyme. To 3.6 μg of native enzyme was added an increasing amount of IgG (either unlabeled or labeled with ^{125}I). The amount of protein in the precipitate was determined by the Lowry method [27] or by radioactivity measurement. ○, % of free enzyme in supernatant (activity determination); ▲, total protein of precipitate (Lowry assay); △, IgG protein of precipitate (from radioactivity measurements); $R = 7$ (5). The molar ratio IgG/enzymes has been calculated at the region of antibody excess from protein and radioactivity (value in the parentheses) estimations of immunoprecipitate.

Fig. 7. Precipitation curve for turnover-modified enzyme. Increasing amounts of IgG were added to 3.4 μg of modified enzyme. ●, % of free modified enzyme (from determination of activity in the presence 3 mM sulfate); ▲, total protein of precipitate (Lowry assay); △, IgG protein of precipitate (radioactivity measurements), $R = 4$ (3). The molar ratio IgG/modified enzyme has been calculated at the region of antibody excess from protein and radioactivity (value in parentheses) estimations of immunoprecipitate.

the immunoprecipitate, but a similar decrease in the ability of turnover-modified enzyme as compared to that of native enzyme to bind IgG was observed independently of which technique was used.

Discussion

The present investigation was carried out with antibodies produced in a goat and directed against immunologic determinants of native arylsulfatase A from rabbit liver. Studies on arylsulfatase A enzymes from mammalian sources indicate the polymeric nature of these enzymes [15,21,31]. The enzyme isolated from rabbit liver exists as a dimer or as a monomer and the aggregation properties are dependent upon pH, protein concentration and ionic strength [11,21]. Because the enzyme is highly dissociated by high pH and low protein concentration [21] the effective antigen is almost certainly the monomer form of arylsulfatase A. Appropriate experiments in the present study could be carried out in the presence of excess antibodies and this had no effect on the results. Thus, the present studies are concerned with the properties of the complex formed by the monomer of arylsulfatase A with antibodies directed against monomer determinants.

Rabbit liver arylsulfatase A is slightly inhibited by the antibody as measured by a reduced activity of the enzyme-antibody complex towards the synthetic substrate nitrocatechol sulfate. The effect of antibodies on the enzymatic activity is probably a function of the relative size of the substrate. Many enzymes with low molecular weight substrates are not significantly inactivated by their specific antibodies, although the activity of the same enzyme towards high molecular weight substrates may be decreased [32]. In the case of rabbit liver arylsulfatase A, the antigenic determinants seem to be located some distance apart from the substrate binding site, since the latter is accessible to the substrate in the antibody-enzyme complex, at least as measured by the K_m value. The K_m value seen with both free enzyme and the enzyme in the complex with antibody was the same at pH 4.5 and also at pH 5.5. Non-identity of the substrate binding site and the antibody combining sites also accounts for the fact that the competitive inhibition constants, K_i, for sulfate and phosphate ions are virtually identical for native arylsulfatase A and for the enzyme in the enzyme-antibody complex (Table I).

Thus, V is the kinetic parameter which is primarily affected by the binding of antibodies to this enzyme. The enzyme-antibody complex cleaved substrate at a slower rate than did free enzyme. The lowering of the rate of substrate hydrolysis seems not to be due to precipitation effects. Inhibition was observed immediately after addition of antibody during the first phase when soluble immune complex is formed, even before incubation at 37° C.

Our data concerning the effects of antibodies on the activity of arylsulfatase A from rabbit liver differ from results presented by Neuwelt et al. [5] for the enzyme isolated from human liver. In their study, the binding of antibody by the enzyme from human liver appeared to cause increases in both K_m and V. That is, the activity of the enzyme was apparently enhanced rather than inhibited upon association with its antibody. Although this might be due to structural differences between the two enzymes, we should point out that

different conditions were used in determining the kinetic properties of enzyme-antibody complex. K_m and V values for the antibody-precipitated human liver enzyme were calculated from kinetic data obtained after a 3 h incubation. Such a long incubation time could have resulted in a selective denaturation or proteolysis of the free enzyme relative to that undergone by enzyme in the antibody complex, thus leading to an apparent increase in V for the complexed enzyme.

The binding of antibodies to the rabbit liver enzyme also changed the pH profile for the hydrolysis of nitrocatechol sulfate. Instead of the two pH optima which are characteristic of the native enzyme [11], the curve for the hydrolysis of nitrocatechol sulfate by enzyme bound to antibodies showed only one rather broad optimum which was shifted towards a slightly higher pH value (Fig. 3). This might be due to the changed kinetic characteristic of the enzyme-antibody complex or the fact that the monomer, which is the only enzyme species bound to the antibody, is the form which is responsible for the pH optimum observed. The present results are consistent with the hypothesis [33] that the biphasic pH optimum curves result from an equilibrium between polymeric forms of arylsulfatase A and that these forms have different pH optima.

At pH 5.5 both the native enzyme and the enzyme-antibody complex show the anomalous kinetics, but because the rate of hydrolysis of nitrocatechol sulfate in the complex is lower than for the free enzyme, the process of inactivation of the enzyme in the immune complex is slower. The inactivation of the enzyme during reaction with nitrocatechol sulfate depends upon the rate of hydrolysis of substrate and upon pH. The conversion of the enzyme from the native state to a modified state is not instantaneous and lowering the initial rate of the enzymic reaction results in a longer $t_{1/2}$, the time required for the velocity to fall to one-half of its initial velocity [20,30].

We have previously described a correlation involving the pH-dependent aggregation properties of the rabbit liver enzyme and the process of inactivation [21]. The data suggested that the monomer, which is the form present at high pH, is the species which undergoes inactivation during substrate cleavage. If this were the only important variable then the enzyme monomer bound to the antibody might be expected to as susceptible to inactivation at pH 4.5 as it is at pH 5.5. However, no significant inactivation was observed with the complex at pH 4.5. The cleavage of substrate by enzyme bound to antibody continued with little reduction in rate, similar to the hydrolysis reaction in a control sample of native enzyme. These results suggest that pH rather than the state of aggregation of the protein is the important variable affecting the inactivation of arylsulfatase A. Native rabbit liver arylsulfatase A is present as a dimer at pH 4.5 while the enzyme bound to antibodies is present as a monomer. Interestingly, the turnover numbers of both of these forms are similar at pH 4.5 (Table I). It has been suggested that exposure of a second binding site or sites is responsible for the so-called anomalous kinetics [18—20]. If this is the case, the present results require this process to be pH-dependent.

In the present study, immunochemical tests have been made in order to compare possible conformation or structural differences between native and modified forms of enzyme. Immunologic saturation of the native enzyme occurred

when 5—7 molecules of IgG were attached to a molecule of monomer. However, the modified enzyme showed a significant reduction in its extent of reactivity with antibodies directed against the native enzyme since only 3—4 molecules of IgG were bound to the turnover-inactivated (modified) enzyme. Even though only an approximate value could be obtained for the number of antigenic determinants using a crude γ-globulin fraction instead of highly purified specific antibodies, there is a definite reduction in the number of IgG molecules bound to the turnover-modified enzyme. This is consistent with a significant covalent modification and/or structural rearrangement of the enzyme during the transformation from the native to the turnover-modified form.

Note added in proof (Received May 30th, 1979)

We have recently demonstrated that the turnover-modified enzyme resulting during the hydrolysis of nitrocatechol sulfate is covalently modified and this sulfation is accompanied by a loss of secondary structure from the protein [34].

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