Preparation and Some Properties of Antibodies with Specificity toward *p*-Nitrophenyl Esters*

Lawrence I. Slobin†

ABSTRACT: Antibodies with specificity toward *p*-nitrophenyl esters were elicited in rabbits by immunization with a *p*-nitrocarbobenzoxy (NZ) conjugate of bovine serum albumin. They were purified with the aid of a NZ conjugate of a 1:1 copolymer of lysine and glutamic acid. Both normal γG-globulin and purified anti-NZ antibodies catalyzed the hydrolysis of *p*-nitrophenylacetate (NPA) according to second-order reaction kinetics. The second-order rate constants (k_2) for the reactions catalyzed by antibody and γG-globulin were approximately equal when (NPA) > (antibody), whereas k_2 was slightly but significantly diminished in the case of antibody when (NPA) < (antibody). The possibility that the hydrolysis of *p*-nitrophenyl esters is inhibited when the ester is present in the antibody active site was confirmed in studies with *p*-nitrophenyl-ε-aminocaproate (ACANE). This active ester was hydrolyzed (according to pseudo-first-order kinetics) about 500 times more rapidly than NPA at pH 7.4 in dilute phosphate buffer. Both γG-globulin and anti-NZ antibodies in the presence of excess "protective" hapten [ε-*N*-(*p*-nitrocarbobenzoxy)aminocaproic acid] slightly accelerated the rate of ACANE hydrolysis. In striking contrast, unprotected antibody markedly inhibited the hydrolytic reaction, the degree of inhibition being proportional to the concentration of antibody.

Most of the physiochemical studies conducted to date on the active sites of antihapten antibodies have examined the interaction between chemically stable small organic molecules and their specific antibodies. Some notable examples, since the classical semiquantitative work of Landsteiner and colleagues (Landsteiner, 1945), are the studies on anti-DNP antibodies by Eisen and co-workers (Farah *et al.*, 1960; Velick *et al.*, 1960), and the work on antilactoside antibodies by Karush (1957, 1962). These investigations have provided important thermodynamic information on the process of antibody–hapten interaction but have yielded few direct clues as to the nature of the chemical environment that comprises the active sites. Recently, a technique of "affinity labeling" antibody active sites has been developed (Wofsy *et al.*, 1962). This technique employs highly reactive diazonium salts which react specifically to form stable covalent derivatives of amino acid residues in certain antibody molecules. By this method it has been possible to label tyrosyl residues in the vicinity of the binding sites of three different antihapten antibodies, including the anti-dinitrophenyl (anti-DNP) system (Fenton and Singer, 1965).

The present investigation was undertaken with the objective of examining the behavior of specific antibodies toward highly reactive *p*-nitrophenyl esters. This class of reagents has been shown to serve as substrates for a variety of enzymes; and one in particular, *p*-nitrophenylacetate, has been extensively studied, both as a substrate for chymotrypsin (Hartley and Kilby, 1954; Kézdy and Bender, 1962), and as an ester whose hydrolysis is catalyzed by nonenzymic proteins (Breslow and Gurd, 1962), peptides, and free amino acids (Koltun *et al.*, 1963). In order to prepare antibodies with the necessary specificity, a new antigenic determinant, the *p*-nitrocarbobenzoxy group, was employed and antibodies to this determinant were purified with the aid of hapten–synthetic polyamino acid conjugates.

Experimental Section

Materials. Analyses were performed by the Analytical Laboratory of the Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel. All melting points were determined in open capillaries and are uncorrected. ε-Aminocaproic acid[1] was obtained from Eastern Chemical Co. (mp 200–202). *p*-Nitrophenylacetate was a gift of Dr. J. Kirsch. After several recrystallizations from absolute ether, it melted at 77.5–

* From the Section of Chemical Immunology, The Weizmann Institute of Science, Rehovoth, Israel. *Received April 26, 1966.* The author was a recipient of a U. S. Public Health Service Fellowship (No. 5-F2-AI-21,023-02) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda 14, Md.

† Present address: Department of Biology, University of California at San Diego, La Jolla, Calif.

[1] Abbreviations used in this work: ACANE, *p*-nitrophenyl-ε-aminocaproate; NZ, *p*-nitrocarbobenzoxy; NZ-ACA, ε-*N*-(*p*-nitrocarbobenzoxy)aminocaproic acid; NZ-(Lys,Glu), *p*-nitrocarbobenzoxy conjugate of a copolymer of lysine and glutamic acid; NPA, *p*-nitrophenylacetate; NZ-BSA, *p*-nitrocarbobenzoxy conjugate of bovine serum albumin; PS buffer, 0.05 M sodium phosphate–0.9% NaCl, pH 7.40. (In the precipitin analysis and the purification procedure the concentration of sodium phosphate was reduced to 0.01 M.)

78.5° and was almost colorless. *p*-Nitrophenylacetic acid was obtained from British Drug Houses, Ltd., and was recrystallized several times from water before use (mp 146–147°). *p*-Nitrocarbobenzoxy chloride was synthesized according to the method of Carpenter and Gish (1952).

A 1:1 copolymer of L-lysine and L-glutamic acid, *p*-(Lys,Glu), was prepared from the *N*-carboxy anhydrides of γ-benzyl L-glutamate (Katchalski and Berger, 1957) and ε-*N*-carbobenzoxy-L-lysine (Katchalski and Sela, 1958) according to Sela *et al.* (1962). The copolymer had an approximate molecular weight of 31,000 based on sedimentation and diffusion in the ultracentrifuge and assumed partial specific volumes of the amino acid residues (Sela *et al.*, 1962). To obtain substitution on approximately one-third of the lysyl residues, the following conditions for carbobenzoxylation were employed. *p*-(Lys-Glu) (1.1 g) was dissolved in a 0.5 N sodium carbonate buffer pH 9.5 (100 ml) and chilled in an ice bath. *p*-Nitrocarbobenzoxy chloride (235 mg) was dissolved in anhydrous dioxane (20 ml) and added by drops to the chilled solution of copolymer with continuous overhead stirring. The addition took approximately 1.5 hr; the stirring was continued an additional 30 min in the cold. The mixture was carefully adjusted to neutral pH with 6 N HCl and dialyzed against 6 l. of deionized water, changed every 12 hr, for 6 days. The slight precipitate that formed in the dialysis bag was removed by centrifugation and the clear supernatant solution lyophilized. The product was stored at 4°. The degree of substitution of the lysyl residues was determined from the molecular weight of the copolymer and the molar extinction coefficient of NZ-Lys at 275 mμ (8.75×10^3 M^{-1} cm^{-1}). Approximately 35% of the lysyl residues bore the nitrocarbobenzoxy group.

The antigen, *p*-nitrocarbobenzoxy-BSA (NZ-BSA), was prepared by treating bovine plasma albumin (Armour, lot no. A69805) with *p*-nitrocarbobenzoxy chloride under conditions identical with those described above for the preparation of NZ-(Lys,Glu). The initial BSA concentration was 2%. To achieve maximal substitution of the lysyl residues of BSA, the weight ratio of protein:NZ-Cl was 5.2:1. After extensive dialysis against deonized water, the protein derivative was obtained as a lyophilized powder and stored at 4° until use. Approximately 80% of the lysyl residues (45 moles of NZ/64,000 g of BSA) reacted, as judged by Van Slyke analysis.

ε-N-(p-Nitrocarbobenzoxy)-L-lysine was synthesized according to the procedure developed for the equivalent carbobenzoxy derivative (Katchalski and Sela, 1958) (mp 238–240°).

Anal. Calcd for $C_{14}H_{19}N_3O_6$: N, 12.91. Found: N, 12.94 (Dumas).

ε-N-(p-Nitrocarbobenzoxy)aminocaproic acid (NZ-ACA) was synthesized according to standard procedures for carbobenzoxylation of amino acids (Greenstein and Winitz, 1961) and the product was recrystallized from water (mp 97–99°).

Anal. Calcd for $C_{14}H_{18}N_2O_6$: N, 9.02. Found: N, 9.05 (Dumas).

p-Nitrophenyl-ε-aminocaproate Hydrobromide (*ACANE·HBr*). The nitrophenyl ester of ε-*N*-(carbobenzoxy)aminocaproic acid was synthesized from ε-*N*-(carbobenzoxy)aminocaproic acid and *p*-nitrophenol by the dicyclohexylcarbodiimide method (Greenstein and Winitz, 1961) using ethyl acetate as a solvent. The corresponding unprotected ester was obtained in 79% yield by removal of the carbobenzoxy blocking group with HBr and glacial acetic acid (Ben-Ishai and Berger, 1952). The product was recrystallized from ethanol–water and dried *in vacuo* over NaOH at 65° (mp 112–114°). Nonaqueous titration in absolute ethanol with sodium methoxide (Patchornik and Ehrlich-Rogozinski, 1959) gave a molecular weight, assuming 2 equiv/mole, of 330 ± 5 (theoretical mol wt 333).

Anal. Calcd for $C_{12}H_{17}BrN_2O_4$: Br, 23.99; N, 8.40. Found: Br, 23.80; N, 8.38.

Normal rabbit γG-globulin[2] was prepared from pooled preimmune sera by ammonium sulfate precipitation and subsequent chromatography on DEAE cellulose using 0.017 M sodium phosphate, pH 6.3, as the eluting buffer (Levy and Sober, 1960).

Methods. IMMUNIZATION PROCEDURE. The NZ-BSA was prepared for injection by the alum precipitation technique (Fleischman *et al.*, 1963) The antigen (10 mg) was administered intramuscularly at weekly intervals for a period of at least 4 weeks. The rabbits were bled from the central ear artery every 5–7 days, commencing 1 week after the last injection. When necessary the animals were boostered by intravenous injection for three consecutive days with 0.5 ml of 0.2% antigen solution in aqueous 0.9% NaCl. During the course of these studies, it was found that antigen incorporated into Freunds adjuvant (Difco Laboratories, Detroit, Mich.) and injected into each footpad greatly increased the response. Antigen in adjuvant was prepared according to Sela *et al.* (1962). Sera having similar antibody titer [as determined by precipitin analysis with NZ-(Lys,Glu)] were pooled and frozen until used. The concentration of anti-NZ antibody in the pooled antisera varied from 0.5 to 1.0 mg/ml.

PRECIPITIN ANALYSIS. Variable amounts of NZ-(Lys-Glu) (usually 10–200 μg in 0.9% NaCl) were added to a constant volume of antiserum (0.25–0.5 ml) and incubated for 1 hr at 37° followed by 1–2 days at 4°. Precipitates were collected by centrifugation, washed three to four times with PS buffer, and air dried. Washed precipitates were dissolved in a suitable volume (usually 5 ml) of alkaline copper reagent and antibody protein estimated by addition of Folin reagent (Fisher Scientific Co.) according to Lowry *et al.* (1951). The NZ-(Lys,Glu) was found to produce only one-seventh the absorbancy of γG-globulin (on a weight basis) at 750 mμ and

[2] The nomenclature used here for immunoglobulins is proposed in *Bull. World Health Organ. 30*, 447 (1964). γG-Globulin denotes that class of immunoglobulins often designated as γ_2- or 7S γ-globulin. Throughout this manuscript the term γG-globulin will refer to protein obtained from nonimmune or normal serum.

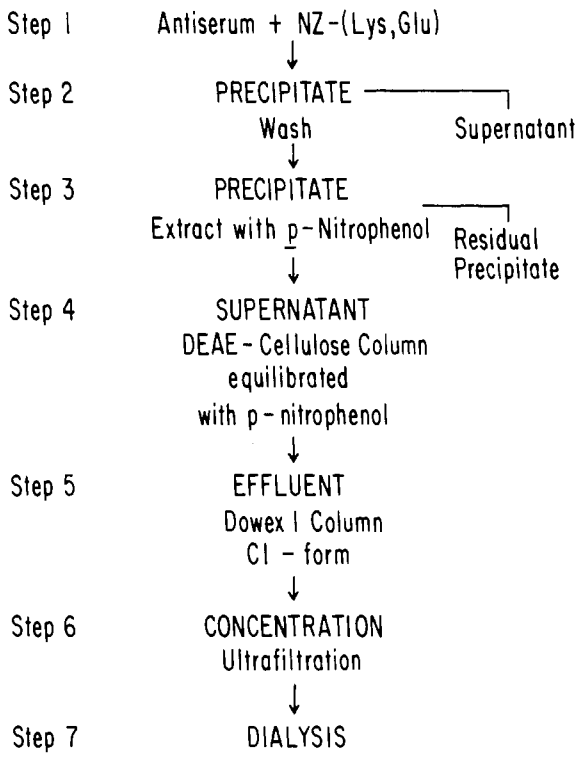


FIGURE 1: Scheme for purification of antibodies specific for the *p*-nitrocarbobenzoxy (NZ) group.

consequently its presence in a specific precipitate made a negligible contribution to the measured optical density.

KINETICS. All pH measurements were made with a Radiometer pH meter, type PHM4C (Radiometer, Copenhagen, Denmark). A phosphate–saline (PS) buffer (0.05 M sodium phosphate, 0.9% NaCl) was employed throughout. The studies with NPA were conducted with a Beckman Model DU spectrophotometer; all other experiments employed the Cary recording spectrophotometer, Model 14. Both spectrophotometers were equipped with thermostated cell compartments (in the Cary, the cell holder was also thermostated) maintained at 25 ± 0.2°.

Solutions (1 ml) containing the appropriate concentration of antibody[3] (or γG-globulin) in the PS buffer were preincubated for 10 min in cuvets of 1-cm path length in the cell holder of the thermostated spectrophotometer and the reagent (NPA or ACANE) introduced in a volume of 2–10 μl by means of a calibrated microsyringe (Hamilton Co., Inc., Whittier, Calif.). The reaction mixture was rapidly stirred with the aid of a polyethylene plunger, and readings were obtained within 15 sec after mixing. In studies with hapten-protected antibody, antibody and protector (NZ-ACA) were preincubated for 10 min and the reaction was initiated by addition of reagent (ACANE). Stock solutions of reagents (ACANE, NPA, and NZ-ACA) were made up in either absolute dioxane or absolute ethanol. The concentration of stock solution was always such that the final concentration of organic solvent was <1%.

The rate of hydrolysis of ACANE or NPA was ordinarily determined by following the rate of appearance of *p*-nitrophenolate anion at pH 7.4 and at 400 mμ. (In studies on the hydrolysis of ACANE as a function of pH, the appearance of NP was followed at 347 mμ, the isosbestic point.) In following the rate of appearance of *p*-nitrophenolate anion, it is necessary to maintain a constant concentration of hydrogen ion since the absorption at 400 mμ measures only the *p*-nitrophenolate anion in an equilibrium mixture with *p*-nitrophenol. Initially, the pH was checked at the conclusion of each run and found to remain unchanged. Thereafter, only occasional checks of pH were made. *p*-Nitrophenolate anion was shown to obey Beer's law over the ranges of concentration studied.

The observed first-order rate constants, k_1, were obtained from the rate of appearance of *p*-nitrophenolate anion using the relation

$$2.303 \log \frac{(OD_\infty - OD_0)}{(OD_\infty - OD_t)} = k_1 t \qquad (1)$$

where t = reaction time in minutes; OD_0 is the optical density at $t = 0$; OD_∞ is the optical density at infinite time, determined after a reaction time of at least 8 half-lives; and OD_t is the optical density at any time t after the start of the reaction. It was established that the hydrolysis of both NPA and ACANE followed pseudo-first-order kinetics by following the reaction continuously for at least 4 half-lives. Thereafter, reactions were followed continuously for at least 2 half-lives.

PURIFICATION PROCEDURE. The scheme employed for the preparation of anti-NZ antibodies is outlined in Figure 1. Except for step 1 (see discussion), it is similar to previously reported methods for the preparation of antihapten antibodies (Farah *et al.*, 1960; Utsumi and Karush, 1964). In a typical moderate scale preparation, 500 ml of clarified antiserum (0.5 mg of antibody/ml) was divided into five equal portions and incubated with an amount of NZ-(Lys,Glu) (22 mg/portion) necessary to give maximal precipitation. After incubation for 2 hr at 37°, the mixture was agitated gently at 4° for 48 hr (step 1). Subsequent steps were performed in the cold unless otherwise noted. The precipitates were collected by centrifugation and washed three times with PS buffer (step 2). The precipitates were then dispersed in 25-ml portions of PS buffer containing 0.1 M *p*-nitrophenol (pH 7.4) and incubated for 1 hr at 37° with gentle stirring (step 3). The pooled supernatant solutions generally contained about 80% of the antibody initially precipitated. *p*-Nitrophenol was chosen to dissociate precipitated antigen–antibody complexes with the aim of selecting antibodies with maximum specificity toward

[3] Concentrations were based on the extinction coefficient at 279 mμ ($E^{1\%}_{1cm}$), taken here as 13.6 for both purified antibody and normal rabbit γG-globulin (see results).

the test substrates and for reasons already cited in studies with anti-DNP antibodies (Farah *et al.*, 1960).

The clear supernatant solutions were pooled and applied to a jacketed column of DEAE-cellulose (35 × 3.1 cm), which previously had been equilibrated with PS buffer containing 0.1 M NP (pH 7.4). Chromatography was performed at 37° using PS buffer containing 0.1 M *p*-nitrophenol (flow rate 1 ml/min). Under these conditions, the solubilized NZ-(Lys,Glu) remained firmly bound to the DEAE-cellulose.

The tubes containing antibody, as determined by Lowry protein (Lowry *et al.*, 1951), were pooled and concentrated by ultrafiltration at room temperature to about 50 ml. The concentrate was passed through a Dowex 1 (Cl^- form, X8, 200–400 mesh) column (47 × 2.4 cm) which was washed and subsequently developed at room temperature (flow rate 0.5 ml/min) with PS buffer (step 5). This step proved effective in removing essentially all the *p*-nitrophenol. However, it is still possible that persistent traces of *p*-nitrophenol remained (Farah *et al.*, 1960). The volume of effluent was reduced by ultrafiltration to give the desired final concentration of antibody (step 6).

After dialysis for 48 hr against several changes of 6 l. of PS buffer, the contents of the dialysis sac was divided into small portions (5 ml) and the antibody stored at −20° (step 7). Small amounts of precipitate occasionally formed during dialysis and were discarded. The amount of protein in the final product was generally about 50% of the antibody present in the initial specific precipitate. The purified antibody gave a single boundary in the ultracentrifuge, sedimenting with $s_{20,w}$ 6.4 S (0.5% solution in PS buffer).

Results

Absorption Spectrum of Purified Anti-NZ Antibodies. In PS buffer purified anti-NZ antibodies had an absorption maximum at 279 mμ and a minimum at 251 mμ. The extinction coefficient ($E^{1\%}_{1cm}$) at 279 mμ was 13.6 (based on Kjeldahl analysis and an assumed N content of 16% (McDuffie and Kabat, 1956), a value identical with anti-DNP antibodies (Farah *et al.*, 1960). The ratio of the absorbancies at $\lambda_{max}:\lambda_{min}$ was in the range 2.5–2.6, a value in good agreement with other purified antihapten antibodies (Farah *et al.*, 1960; Karush and Marks, 1957). The purified antibodies did not absorb at 400 mμ at concentrations <0.5%. These spectral analyses indicate that gross contamination of antibody preparations with ultraviolet absorbing impurities (*e.g.*, antigen or *p*-nitrophenol) was unlikely.

Specific Precipitability and Dissolution of Specific Precipitates by Haptens. Whereas anti-(NZ-BSA) antiserum precipitated both BSA and NZ-(Lys,Glu) as well as NZ-BSA (Figure 2), purified antibodies reacted only with homologous antigen and the copolymer derivative. If the total antibody precipitated from antiserum by NZ-BSA is taken as 100%, then NZ-(Lys,-Glu) and BSA precipitated 45 and 30%, respectively, of the total. Absorption of antiserum with NZ-(Lys,Glu)

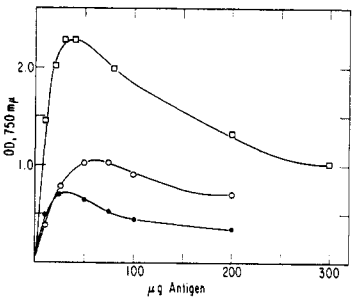


FIGURE 2: Extinction, at 750 mμ, of dissolved precipitates (treated with alkaline copper and Folin reagents according to Lowry *et al.*, 1951) obtained by the addition of NZ-BSA (□), NZ-(Lys,Glu) (○), and BSA (●) to the antiserum to NZ-BSA, as a function of the amount of the precipitant. With both BSA and NZ-(Lys,Glu), 0.5 ml of antiserum was used. In the case of NZ-BSA, 0.25 ml of antiserum was used and the observed optical density at 750 mμ was multiplied by 2.

followed by absorption with BSA still left approximately 20% of the antibody initially present free to react with NZ-BSA. Specific precipitation of the purified protein with NZ-(Lys,Glu) or NZ-BSA varied between 75 and 90%.

Figure 3 shows the effect of various added haptens on the solubility of specific precipitates formed from unfractionated antiserum. If the effect on solubility of precipitates is taken as a crude measure of the affinity of hapten for anti-NZ antibodies, then it may be seen that ε-*N*-(*p*-nitrocarbobenzoxy-L-lysine) had the greatest affinity; *p*-nitrophenylacetic acid had somewhat less affinity and *p*-nitrophenol proved least effective in causing dissolution. These results are exactly analogous to those obtained by Farah *et al.* (1960) with anti-DNP antibodies.[4]

Reaction of γG-Globulin and Anti-NZ Antibodies with p-Nitrophenylacetate. It was found that, as in the case with several nonenzymic proteins (Hartley and Kilby, 1954; Breslow and Gurd, 1962), γG-globulin is capable of catalyzing the hydrolysis of NPA. The observed rate of hydrolysis obeyed pseudo-first-order kinetics up to a reaction time of 4 half-lives. A calculated second-order rate constant of 270 ± 7 l. $mole^{-1}$ min^{-1} was obtained from a plot of k_1 *vs.* concentration of γG-globulin in mg/ml[5] (Figure 4). As a check on this treatment of the data, the extrapolated value of k_1 at zero concentration of γG-globulin, which should be equal to the spontaneous rate of hydrolysis due to solvent (H_2O), was found to be 1×10^{-3} min^{-1}. This value is in excellent agreement with a previous deter-

[4] These investigators found that the following haptens, in order of effectiveness, were capable of dissolving specific precipitates of anti-DNP antibodies: ε-DNP-L-lysine, DNP-glycine, dinitrophenylacetate, dinitrophenol.

[5] The molecular weight of both γG-globulin and purified antibody was taken as 1.5×10^5.

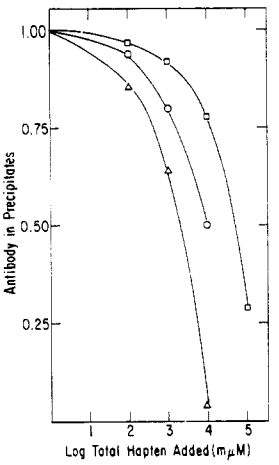


FIGURE 3: Dissolution of specific precipitates by hapten addition to unfractionated antiserum. Dissolution was carried out as follows: 1.0 ml of pooled anti-(NZ-BSA) antiserum and 100 μg of NZ-(Lys,Glu) (76 mμequiv of NZ) in 1.0 ml of PS buffer were incubated for 60 min at 37° and for 48 hr at 4°. Precipitates were washed twice with ice-cold PS buffer, resuspended in 1 ml of PS buffer containing variable amounts of hapten, and incubated for 60 min at 37°. Remaining precipitate was again washed twice with PS buffer, allowed to dry *in vacuo* over P_2O_5 overnight, and analyzed. Three control precipitates (unexposed to hapten) gave an optical density at 750 mμ of 1.420, 1.370, and 1.435 corresponding to approximately 300 μg of antibody. The amount of antibody in precipitates is given on the ordinate as a fraction of the amount present in control precipitates. Dissolution by ε-*N*-(*p*-nitrocarbobenzoxy-L-lysine) (△); dissolution by *p*-nitrophenylacetic acid (○); dissolution by *p*-nitrophenol (□).

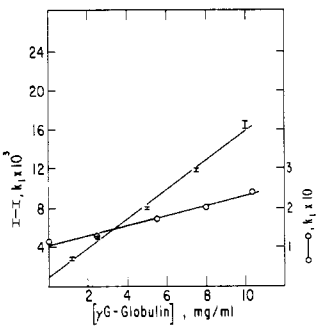


FIGURE 4: Plot of the observed first-order rate constant (k_1) for the γG-globulin catalyzed hydrolysis of NPA (I) and ACANE (○) at 25° in PS buffer pH 7.40 *vs.* concentration of γG-globulin in milligrams per milliliter. Extremities of bar (I) represent values obtained in duplicate determinations.

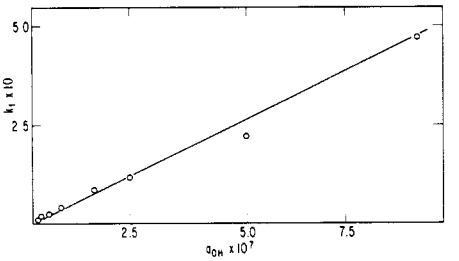


FIGURE 5: Plot of the observed first-order rate constant (k_1) for the hydrolysis of ACANE at 25° in PS buffer as a function of hydroxyl ion activity.

mination of the first-order rate constant for the hydrolysis of NPA in water at pH 7.4 (Koltun *et al.*, 1958).

When the rate of hydrolysis of NPA was measured in the presence of anti-NZ antibodies an almost identical second-order rate constant was obtained. At an antibody concentration of 10 mg/ml (6.65×10^{-5} M) and at an initial concentration of 10^{-4} M NPA, the calculated second-order rate constant was 300 ± 30 l. mole^{-1} min^{-1} for three different antibody preparations. When the ratio of antibody to NPA concentration was increased ((antibody) = 6.67×10^{-5} M; (NPA) = 5×10^{-5} M]), the rate constant was lowered somewhat to a value of 215 ± 20 l. mole^{-1} min^{-1}. Changing the initial ratio of reactants had no effect on the kinetic constant for γG-globulin catalysis. These results indicated that (1) antibodies with specificity toward the NZ group did not enhance the rate of hydrolysis of NPA compared with γG-globulin, and (2) that specific antibody appears to slightly retard the hydrolytic reaction (compared again with γG-globulin) when the rate is measured in antibody excess, *i.e.* (antibody)/(NPA) > 1.[6]

Hydrolysis of p-Nitrophenyl-ε-aminocaproate. In order to test the possibility that specific antibodies can retard (or quench) the hydrolysis of "active" esters, it was necessary to obtain a *p*-nitrophenyl ester that would (1) bind to antibody active sites, (2) display a much greater "spontaneous" reactivity at neutral pH than NPA, and (3) react sluggishly or not at all with γG-globulin. For this purpose ACANE was synthesized and found to possess both the desired specificity and reactivity. The rate of *p*-nitrophenol liberation was linearly dependent on a_{OH} (calculated from pH and $K_w = 10^{-14}$) between pH 6.0 and 8.0 (Figure 5). In PS buffer, the rate of ACANE hydrolysis followed pseudo-first-order kinetics with a k_1 of 1.15×10^{-1} min^{-1} at pH 7.40 and 25° (Figure 6), and k_1(ACANE)/k_1(NPA) was approximately 500. Since the intercept in Figure 5 at zero hydroxide ion concentration is zero, the hydrolysis of ACANE appears to be catalyzed specifically by hydroxyl ion.

$$\frac{\text{d(nitrophenol)}}{dt} = k_{OH}(OH^-)(\text{ACANE}) \qquad (2)$$

[6] The concentration of antibody active sites is presumably twice the molar concentration of antibody.

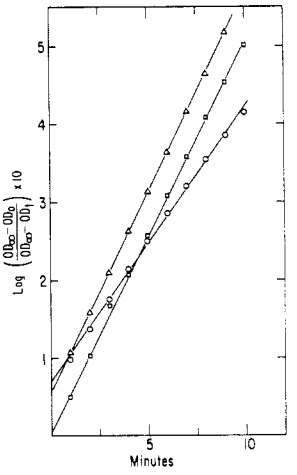


FIGURE 6: First-order plots for the hydrolysis of ACANE (5×10^{-5} M) at pH 7.40 and 25° in the presence of PS buffer (□), in the presence of 3.5×10^{-5} M anti-NZ antibodies in PS buffer (○), and in the presence of 3.5×10^{-5} M anti-NZ antibodies plus 4×10^{-4} M NZ-ACA (△).

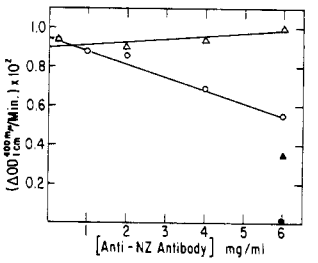


FIGURE 7: Initial velocities (ΔOD (400 mμ, 1 cm)/min) for the hydrolysis of ACANE at pH 7.40 and 25° as a function of the concentration in milligrams per milliliter of hapten-protected (plus 4×10^{-4} M NZ-ACA) and free (minus NZ-ACA) anti-NZ antibodies. △, 1×10^{-5} M ACANE and hapten-protected antibodies; ○, 1×10^{-5} M ACANE and free antibodies; ▲, 5×10^{-6} M ACANE and hapten-protected antibodies; ●, 5×10^{-6} M ACANE and free antibodies.

TABLE I: Kinetic Constants for the Hydrolysis of 5×10^{-5} M ACANE in the Presence of Anti-NZ Antibodies at pH 7.40 and 25°

Concn of Anti-NZ Antibodies (M) × 10^5	Concn of NZ-ACA (M) × 10^4	k_1 (min^{-1}) × 10
5.3	...	0.60
3.5	...	0.81
1.8	...	1.09
0.70	...	1.15
1.8	5	1.19
7.0	5	1.45

The slope of Figure 5 gives a specific rate constant for hydroxyl ion catalysis of 5×10^5 l. $mole^{-1}$ min^{-1}. By ascending paper chromatography (using 1-butanol–propanol–water, 10:10:5 as a solvent; Czerepko, 1958), the products of the reaction were identified as ε-aminocaproic acid and *p*-nitrophenol.

Reaction of γG-Globulin and Anti-NZ Antibodies with p-Nitrophenyl-ε-aminocaproate. The hydrolysis of ACANE, like that of NPA, was catalyzed by γG-globulin (Figure 4). However, because of the high "spontaneous" reactivity of ACANE compared with NPA, γG-globulin had relatively little effect on the observed first-order rate constant. For example, the ratio of k_1 for the hydrolysis of NPA in the presence of 10 mg/ml of γG-globulin to k_1 in buffer solution at the same pH (7.4) was 16.4:1 compared with a ratio of 2.1:1 for the hydrolysis of ACANE under identical conditions. A fortunate feature of this result is that it permitted the study of the effect of antibody concentration on ACANE hydrolysis under conditions in which increasing concentrations of hapten-protected antibody had only a slight effect on the initial rate of *p*-nitrophenol formation (Figure 7).

The hydrolysis of ACANE in the presence of anti-NZ antibodies appeared to follow pseudo-first-order kinetics (Figure 6). However, the rate was diminished compared with either γG-globulin or antibody to which a protective hapten (NZ-ACA) was added in large excess of antibody active site concentration[7] (Table I). Anti-BSA, isolated from anti-(NZ-BSA) antiserum (Freedman *et al.*, 1966) after absorption with NZ-(Lys,Glu), behaved identically with hapten-protected antibody. The absorption spectrum and extinction at 400 mμ of *p*-nitrophenol remained unaltered in the presence of antibody excess, thus eliminating the possibility that the observed inhibition was due to some change in the spectral properties of *p*-nitrophenol. After completion of the hydrolytic reaction (6 half-lives), antibody (5.3×10^{-5} M) was retreated with ACANE (5×10^{-5} M). The observed first-order rate constant (0.65×10^{-1} min^{-1}) was almost identical with the one obtained with previously unreacted antibody.

In order to more definitely establish the retarding effect of anti-NZ antibodies on the hydrolysis of ACANE, the following experiment was performed.

[7] Hapten-protected antibodies appeared to have less effect on ACANE hydrolysis than γG-globulin. For example, at equivalent concentrations (3.5×10^{-5} M) of protein, the observed first-order rate constant for γG-globulin was 2.0×10^{-1} min^{-1} compared with a k_1 value of 1.45×10^{-1} min^{-1} for anti-NZ antibodies. This difference may be due either to incomplete protection by NZ-ACA, or to slight contamination of γG-globulin with an enzyme with trypsinlike specificity. It was found by the author that ACANE hydrolysis was catalyzed by trypsin.

The initial rate of *p*-nitrophenol formation at a *fixed* concentration of ACANE was determined at increasing concentrations of antibody both in the presence and absence of an excess of NZ-ACA. (In a separate experiment, it was found that NZ-ACA had no effect on the rate of ACANE hydrolysis.) The results are shown in Figure 7. They demonstrate that the rate of ACANE hydrolysis is progressively inhibited by increasing concentrations of anti-NZ antibody. In fact, if the molar ratio of antibody to ACANE is great enough (Figure 7, solid circles), the initial velocity becomes too slow to measure. In striking contrast, increasing the amount of hapten-protected antibody serves to slightly accelerate the rate of reaction.

Discussion

Purification Procedure. The one novel feature of the purification procedure was the use of hapten–synthetic polyamino acid conjugates to precipitate antibodies to a hapten–protein conjugate. The possible advantages of this procedure deserve some discussion. Most organic "active" groups which are employed to conjugate haptens to protein carriers react with a variety of amino acid residues commonly found in proteins. For example, diazonium ions, which are commonly used to make hapten–protein conjugates, can react with tyrosyl, histidyl, and lysyl residues (Tabachnick and Sobotka, 1960).

As already pointed out by Singer (1964), haptens are usually attached to protein carriers on a variety of amino acid side chains. These side chains may, in turn, be located in regions of the protein molecule which possess different secondary structures. Furthermore, it is quite likely that an antihapten antigenic determinant involves at least the amino acid side chain to which the hapten is attached (Farah *et al.*, 1960) and possibly adjoining amino acid residues. Thus, the use of a heterologous precipitating protein, *e.g.*, anti-(X-BSA) antiserum treated with X-(γG-globulin), to initiate antibody purification may well result in the isolation of antibodies with specificities toward a variety of hapten–amino acid residue conjugates as well as to complex structural constellations, of hapten and adjacent amino acid residues, that may be shared by both proteins (Singer, 1964). The use of synthetic polyamino acid–hapten conjugates to initiate the purification of an antihapten antibody may offer a way of selecting a population of antibodies with specificity mainly (or exclusively) toward one amino acid residue–hapten conjugate.

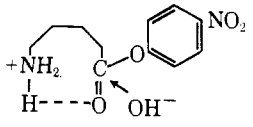
It was presumed in this investigation that *p*-nitrocarbobenzoxy chloride reacted solely with ε-amino groups of the lysyl residues in BSA although reactions with other amino acid side chains are possible (Olcott and Fraenkel-Conrat, 1947). However, the results shown in Figure 2 indicate, as often observed before (Haurowitz and Schwerin, 1943), that antiserum against NZ-BSA contained antibody specificities other than those directed against BSA or NZ-Lys. Purified antibodies, on the other hand, reacted solely and to the same extent with NZ-BSA and NZ-(Lys,Glu). Comparison by direct binding studies, employing synthetic polyamino acids and proteins (both conjugated with hapten) to initiate antibody purification, should establish whether the use of synthetic hapten carriers results in a population of antibodies with more narrowly defined specificities.

Reaction of p-Nitrophenylacetate with γG-Globulin and Anti-NZ Antibodies. *p*-Nitrophenylacetate has been shown to react specifically with a variety of enzymes resulting in the acetylation of (1) a single serine residue of chymotrypsin (Hartley and Kilby, 1954; Balls and Wood, 1956); (2) three cysteine residues of glyceraldehyde 3-phosphate dehydrogenase (Park *et al.*, 1961; Cunningham and Schepman, 1963); and (3) at least two as yet unidentified amino acid residues in creatine phosphokinase (Clark and Cunningham, 1965). These specific reactions are usually characterized by a "burst" of *p*-nitrophenol and this accelerated rate of hydrolysis has been shown in the case of chymotrypsin and other enzymes to be due to reaction with the active site(s). In contrast, NPA hydrolysis is nonspecifically catalyzed by a variety of nonenzymic proteins (Hartley and Kilby, 1954; Breslow and Gurd, 1962), as well as peptides and free amino acids (Koltun *et al.*, 1963). In the case of the nonspecific reactions, the rate of *p*-nitrophenol formation usually follows pseudo-first-order kinetics at a given concentration of protein (or peptide), and the over-all reaction is characterized by a defined second-order rate constant.

The kinetic results obtained here with rabbit γG-globulin and NPA may be described by a second-order rate constant, and therefore the reaction appears to be of the nonspecific variety (Figure 4). At the pH (7.4) employed in these studies, the nonspecific catalysis may presumably be ascribed to the imidazole group of the histidyl residues of γG-globulin, the free imidazole being regenerated by the spontaneous decomposition of acylimidazole (Breslow and Gurd, 1962; Koltun *et al.*, 1963).

When anti-NZ antibodies were treated with NPA at a molar ratio of antibody:NPA of 0.67:1, they appeared to behave identically with γG-globulin indicating that there are no residues in the specific antibody with unusual reactivity toward NPA. That an interaction between NPA and antibody active sites had occurred was revealed by a decrease in the second-order rate constant by a factor of 0.72 when the initial molar ratio of antibody:NPA was increased to 1.33:1. No such change in rate constant was observed when the initial ratios of NPA and γG-globulin were varied. The dissolution of specific immune precipitates by *p*-nitrophenol (Figure 2) also makes it appear likely that an interaction between NPA and anti-NZ antibodies does occur. The inhibition of NPA hydrolysis may be explained by the absence of *effective* nucleophilic groups in the active sites of anti-NZ antibodies. Thus, if there are any "catalytic" groups present in antibody active sites,[8] a possibility not eliminated by this study, they are not revealed by their reactivity toward *p*-nitrophenylacetate.

Hydrolysis of p-Nitrophenyl-ε-aminocaproate. The solvolysis of ACANE in dilute phosphate buffer between pH 6 and 8 appears to be an example of specific hydroxyl ion catalysis (Figure 5). Paper chromatography of the reaction products indicated that ε-aminocaproic acid and *p*-nitrophenol are formed and although other reaction products are conceivable, it appears unlikely that the hydrolysis proceeds *via* the formation of the seven-membered ε-aminocaprolactam. Lactam formation has previously been observed in the hydrolysis of some esters of γ-(4-imidazolyl)butyric acid (Bruice and Sturtevant, 1959). In the case of the *p*-nitrophenyl ester, these workers indicated that, in addition to an intramolecular mode of catalysis leading to lactam formation, a very large second-order rate of reaction with hydroxide ion was encountered. They postulated two mechanisms to explain the assistance of the imidazole group on the solvolysis of these substituted esters of butyric acid. One of the mechanisms involved the intramolecular solvation of the transition state for OH^- attack by the imidazolium ion. In this case, it is possible that the enhanced rate of hydrolysis of ACANE compared with NPA at pH 7.4 is due to the intramolecular solvation of the transition state by NH_3^+ ion.



As further support for the above mechanism it is known that ε-aminocaproic acid ($pK_1' = 4.43$) is a stronger acid than *n*-caproic acid ($pK_1' = 4.85$). Regardless of the detailed mechanism, it seems likely that some kind of anchimeric assistance by NH_3^+ is involved in the solvolysis of ACANE.

Reaction of ACANE with Anti-NZ Antibodies. The technique of hapten dissolution has previously been employed as a simple means of estimating, in relative terms, antibody–hapten affinities (Farah *et al.*, 1960). From experiments employing this technique (Figure 3), it was expected that ACANE, which by virtue of the *p*-nitrophenyl group and the long aliphatic side chain resembles ε-*N*-(*p*-nitrocarbobenzoxy)-L-lysine, would bind to anti-NZ antibodies even more effectively than NP or NPA.

Several experiments indicate that the hydrolysis of ACANE is specifically inhibited when the ester is present in antibody combining sites. First, the observed first-order rate constant is decreased in the presence of antibody (Figure 6 and Table I). This decrease is offset by incubation of antibody with a large excess of protective hapten (NZ-ACA) prior to treatment with ACANE. Hapten-blocked antibody behaved similarly to both γG-globulin and anti-BSA isolated from the same antiserum used to prepare anti-NZ-BSA. A similar type of hapten protection experiment has been employed to establish the specificity of reagents used to affinity-label antibody active sites (Wofsy *et al.*, 1962).

When ACANE at a given concentration was incubated with anti-NZ antibody at increasing concentrations, the initial rate of hydrolysis was considerably diminished. This effect was also reversed by prior blocking of active sites with NZ-ACA (Figure 7). Incubation of ACANE with antibody under conditions of large antibody excess reduced the rate of hydrolysis so that it became too slow to measure conveniently (Figure 7, solid circles).

A possible explanation for the observed inhibition of ACANE hydrolysis involves a decrease in the rotational mobility of antibody-bound ester. When present in an antibody active site, the flexibility of the aliphatic tail of ACANE may be sufficiently restricted so as to preclude the possibility of anchimeric assistance of the ε-amino group in the solvolytic reaction. In this situation, ACANE hydrolysis would proceed at a rate comparable to that of NPA, *i.e.*, at a rate too slow to be conveniently measured. (The half-life for the hydrolysis of NPA at pH 7.4 in dilute phosphate buffer is approximately 11 hr.)

Recently, Stryer and Griffith (1965) have made an electron spin resonance study of the interaction of anti-DNP antibodies with a spin-labeled hapten, dinitrophenyl "nitroxide." The striking changes observed in the electron spin resonance spectrum upon addition of the antibody indicated that the tumbling motion of the nitroxide free radical is markedly reduced upon formation of an antibody–hapten complex. The authors concluded that antibody combining sites are characterized by a high degree of rigidity. Such rigidity of the antibody combining sites might well account for the kinetic effects reported here. Although several alternative explanations may be offered, the author feels more detailed study of the hydrolysis of antibody-bound ACANE is necessary before any detailed mechanism of inhibition is established.

The possibility of studying other hydrolytic reactions that are affected by the presence of antibody is envisaged. Such a kinetic approach may help to explore aspects of hapten–antibody interactions that are not revealed by more conventional thermodynamic binding studies.

Acknowledgment

The author is indebted to Professor Michael Sela, in whose laboratory this work was performed, for his warm hospitality, advice, encouragement, and for reading this manuscript prior to publication. Thanks are also due to Mr. I. Jacobson for his aid with the synthesis and to the numerous members of the Section of Chemical Immunology and the Department of Biophysics of the Weizmann Institute who generously gave of their time to comment on and assist in various phases of this work.

[8] Tyrosyl residues appear to be definitely associated with a variety of antibody active sites (Fenton and Singer, 1965). Although other amino acid residues bearing functional groups, *e.g.*, histidine, are undoubtedly involved in conferring specificity to antibody active sites, their presence has not yet been directly demonstrated.

References

Balls, A. K., and Wood, H. N. (1956), *J. Biol. Chem. 219*, 245.

Ben-Ishai, D., and Berger, A. (1952), *J. Org. Chem. 17*, 1564.

Breslow, E., and Gurd, F. R. N. (1962), *J. Biol. Chem. 237*, 371.

Bruice, T. C., and Sturtevant, J. M. (1959), *J. Am. Chem. Soc. 81*, 2860.

Carpenter, F. H., and Gish, D. T. (1952), *J. Am. Chem. Soc. 74*, 3818.

Clark, J. R., and Cunningham, L. (1965), *Biochemistry 4*, 2637.

Cunningham, L. W., and Schepman, A. M. (1963), *Biochim. Biophys. Acta 73*, 406.

Czerepko, K. (1958), *Mikrochim. Acta*, 638.

Farah, F. S., Kern, M., and Eisen, H. N. (1960), *J. Exptl. Med. 112*, 1195.

Fenton, J. W., II, and Singer, S. J. (1965), *Biochem. Biophys. Res. Commun. 20*, 315.

Fleischman, J. B., Porter, R. R., and Press, E. M. (1963), *Biochem. J. 88*, 220.

Freedman, M. H., Slobin, L. I., Robbins, J. B., and Sela, M. (1966), *Arch. Biochem. Biophys.* (in press).

Greenstein, J. P., and Winitz, M. (1961), "Chemistry of the Amino Acids," Vol. 2, New York, N. Y., Wiley, Chapter 10.

Hartley, B. S., and Kilby, B. A. (1954), *Biochem. J. 56*, 288.

Haurowitz, F., and Schwerin, P. (1943), *J. Immunol. 47*, 111.

Karush, F. (1957), *J. Am. Chem. Soc. 79*, 3380.

Karush, F. (1962), *Advan. Immunol. 2*, 1.

Karush, F., and Marks, R. (1957), *J. Immunol. 78*, 296.

Katchalski, E., and Berger, A. (1957), *Methods Enzymol. 3*, 546.

Katchalski, E., and Sela, M. (1958), *Advan. Protein Chem. 13*, 243.

Kézdy, F. J., and Bender, M. L. (1962), *Biochemistry 1*, 1097.

Koltun, W. L., Dexter, R. N., Clark, R. E., and Gurd, F. R. N. (1958), *J. Am. Chem. Soc. 80*, 4188.

Koltun, W. L., Ng, L., and Gurd, F. R. H. (1963), *J. Biol. Chem. 238*, 1367.

Landsteiner, K. (1945), "The Specificity of Serological Reactions," Cambridge, Mass., Harvard Univ.

Levy, H. B., and Sober, H. A. (1960), *Proc. Soc. Exptl. Biol. Med. 103*, 250.

Lowry, O. H., Rosebrough, N. J., Farr, L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

McDuffie, F. C., and Kabat, E. A. (1956), *J. Immunol. 77*, 193.

Olcott, H. S., and Fraenkel-Conrat, H. (1947), *Chem. Rev. 41*, 151.

Park, J. H., Meriwether, B. P., Clodfelder, P., and Cunningham, L. W. (1961), *J. Biol. Chem. 236*, 136.

Patchornik, A., and Ehrlich-Rogozinski, S. (1959), *Anal. Chem. 31*, 985.

Sela, M., Fuchs, S., and Arnon, R. (1962), *Biochem. J. 85*, 223.

Singer, S. J. (1964), *Immunochemistry 1*, 15.

Stryer, L., and Griffith, O. H. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1785.

Tabachnick, M., and Sobotka, H. (1960), *J. Biol. Chem. 235*, 1051.

Utsumi, S., and Karush, F. (1964), *Biochemistry 3*, 1329.

Velick, S. F., Parker, C. W., and Eisen, H. N. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 1470.

Wofsy, L., Metzger, H., and Singer, S. J. (1962) *Biochemistry 1*, 1031.