Recovery of Antibody Activity upon Reoxidation of Completely Reduced Polyalanyl Heavy Chain and Its Fd Fragment Derived from Anti-2,4-dinitrophenyl Antibody*

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ABSTRACT: Immunospecifically isolated high-affinity antibodies to the 2,4-dinitrophenyl group were mildly reduced, alkylated, and alanylated, and the heavy chains, enriched with 83 DL-alanine residues per molecule, were separated by gel filtration. An active poly-DL-alanyl Fd fragment, which is the N-terminal moiety of the heavy chain, was prepared by the cleavage of the parent poly-DL-alanyl heavy chain with cyanogen bromide in 0.05 N HCl.

The isolated poly-DL-alanyl heavy chain and poly-DL-alanyl Fd fragment were completely soluble in neutral aqueous buffer. The number of hapten binding sites was 0.26 for the alanylated heavy chain and 0.16 for the alanylated Fd fragment. The average association constant for both of them was the same, 2.4×10^6 M^{-1}. This value is two orders of magnitude lower than that of the intact antibody. The recovery of hapten binding sites after complete reduction in 8 M guanidine hydrochloride, followed by reoxidation, was 37 and 59% for the poly-DL-alanyl heavy chain and the poly-DL-alanyl Fd fragment, respectively. The average association constant of the above reoxidized samples was around 1×10^6 M^{-1}, which was of the same order of magnitude as that of the untreated materials. The antibody activity of the heavy chain and of its N-terminal moiety was recovered, thus, in a high yield, even though the reoxidation was performed in the absence of the light chain. This suggests that the conformation of the combining site in the heavy chain is solely dependent upon its own primary amino acid sequence.

The hypothesis that the sequence of amino acids in protein solely determines their three-dimensional structure (Anfinsen, 1962, 1967; Epstein *et al.*, 1963) has been verified for a large number of biologically active proteins by demonstrating the renaturation of their completely reduced and unfolded polypeptide chains (for references, see Neumann *et al.*, 1967). This was extended recently to the antigenic properties of immunoglobulin G.[1] All antigenic determinants of rabbit IgG are conformation dependent, so that the total reduction of the poly-DL-alanyl rabbit IgG led to total loss of reaction with goat antibodies to rabbit IgG, whereas most of the antigenic activity was recovered upon reoxidation (Freedman and Sela, 1966a).

In all the above cases the opening of all the disulfide bridges in the protein molecule resulted in complete loss of its original native structure and of the biological activity. Disulfide bonds do not contribute, nevertheless, exclusively to the stabilization of protein molecules, as is obvious from the abundance of proteins devoid of cystine but still possessing unique conformations. In the case of antibodies of the IgG class, which contain many disulfide bonds, it is possible to open all the interchain bridges without impairing the antibody activity (Weir and Porter, 1966).

The problem of the recovery of antibody activity after total reduction of all the disulfide bonds in the molecule, followed by reoxidation, has been of special interest in view of its impact on theories of antibody formation. Significant recovery of activity was obtained both for the Fab fragments of antibody (Haber, 1964; Whitney and Tanford, 1965) and for a poly-DL-alanyl antibody (Freedman and Sela, 1966b).

Immunoglobulins of the IgG class are composed of two types of chains, two heavy and two light chains. It is not clear yet whether the antibody combining site is present in only one of the chains or whether both chains are required for the formation of this site. Various experiments have demonstrated that isolated heavy chains still contain part of the antibody activity (reviewed by Fleischman, 1966; Porter and Weir, 1966) as well as preserve the specificity of binding present in the original antibody (Haber and Richards, 1966). On the other hand, the activity of the heavy chains is increased significantly upon the addition of light chains, which leads to the noncovalent association of heavy and light chains (Olins and Edelman, 1964; Roholt *et al.*, 1965a).

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[1] The nomenclature for immunoglobulins used is that recommended by the World Health Organization (*Bull. World Health Organ. 30*, 447 (1964)); abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: $(Ala)_n$, poly-DL-alanyl; phosphate-buffered saline, 0.15 M NaCl–0.01 M sodium phosphate buffer (pH 7.3).

This increase in activity could be obtained even if nonspecific light chains are used, but it is always higher if light chains from an antibody are added to heavy chains of the same specificity (Edelman *et al.*, 1963; Roholt *et al.*, 1964; Fraňek *et al.*, 1965; Weir and Porter, 1966). On the other hand, even when the light chains are derived from antibodies of identical specificity, the re-formation is more successful when the light chain was isolated from the same animal as the heavy chain (Roholt *et al.*, 1965b).

Thus, it is not clear if the light chains play a specific role in the active antibody site together with heavy chains, or whether their role is only to stabilize the site. Whatever the case, it is of interest to elucidate whether their presence is necessary at the time of the formation of the active site on the heavy chain. In the experiments mentioned above on the recovery of antibody activity after complete reduction and unfolding (Haber, 1964; Whitney and Tanford, 1965; Freedman and Sela, 1966b), always both chains were present in the reoxidation mixture (in the experiments employing Fab fragments, light chains and Fd fragment, which is the N-terminal half of heavy chain, were present). It was possible, therefore, that in these experiments the recovery of antibody activity was due to an interplay between the two chains, and that heavy chains alone, in the absence of light chains, might not be able to re-form any combining activity after complete reduction and unfolding. In order to investigate this possibility, we performed the oxidation of fully reduced and unfolded heavy chains or Fd fragments, derived from poly-DL-alanyl antibody to the 2,4-DNP groups. The purpose of the poly-DL-alanylation was to obtain the heavy chain in a form well soluble in water (Fuchs and Sela, 1965) as well as to prepare water-soluble products upon complete reduction (Freedman and Sela, 1966a,b). The results clearly demonstrate that isolated heavy chains or Fd fragments, free of light chains, which have been completely reduced in 8 M guanidine hydrochloride could, upon reoxidation, regain a significant amount of the antibody activity present before the reduction.

Experimental Section

Materials. Guanidine hydrochloride (Eastman Organic Chemicals) was recrystallized from absolute ethanol. 2-Mercaptoethanol (Eastman Organic Chemicals) was redistilled before use. Iodoacetamide (Fluka, Switzerland) was recrystallized twice from ethyl acetate–petroleum ether. 2,4-DNP-OH was recrystallized three times from boiling water. 2,4,6-Trinitrobenzenesulfonic acid was obtained from Eastman Organic Chemicals. ε-DNP-L-lysine was obtained from Yeda, Israel. [^{3}H]Acetic anhydride (400 mCi/mmole) was from New England Nuclear Corp. Dowex AG1-X8 (200–400 mesh, Cl^-) was obtained from Bio-Rad Laboratories. Sephadex G-75, G-100, and G-200 was purchased from Pharmacia, Uppsala, Sweden. Crystalline bovine serum albumin was obtained from Armour, Chicago, Ill. Urea (analytical reagent grade) was from British Drug Houses.

Preparation of Antigens. Hemocyanin was prepared from the hemolymph of the crab *Calinectes pelagicus* and was purified on a Sephadex G-200 column. DNP-hemocyanin was prepared according to Eisen *et al.* (1953), using 2,4-dinitrobenzenesulfonate in a weight ratio of 1:3 to hemocyanin. The DNP-hemocyanin contained 10 moles of DNP/100,000 g of hemocyanin. DNP-bovine serum albumin was similarly prepared except that a ratio of 1:10 of 2,4-dinitrobenzenesulfonate to bovine serum albumin was used.

Immunization Procedure. In order to obtain high-affinity antibodies, small doses of the immunogen were injected (Eisen, 1964a). Rabbits were given one injection (2 mg) of DNP-hemocyanin into multiple intradermal sites and hind footpads, using an emulsified mixture composed of 1 ml of DNP-hemocyanin in 0.15 M NaCl and 1.5 ml of complete Freund's adjuvant. Three months later a second injection of 0.5 mg of the immunogen was given according to the same immunization procedure. Animals were bled 15 days after the second injection and sera were collected twice a week and were pooled. The antibody content of the antisera was 1.5–2 mg/ml, as measured by the precipitin technique (Kabat and Mayer, 1961) using DNP-bovine serum albumin as the test antigen.

Preparation of Immunoadsorbent. Bromoacetylcellulose was prepared according to Robbins *et al.* (1967) and was stored at 4°. Bovine serum albumin (0.8 g) was treated with bromoacetylcellulose (3 g) as described by Robbins *et al.* (1967). The bovine serum albumin–bromoacetylcellulose conjugate was washed five times with phosphate-buffered saline and twice with 0.5% Na_2CO_3. The washed conjugate was suspended in 100 ml of 0.5% Na_2CO_3, and 0.5 g of TNP-benzenesulfonic acid, neutralized with 1% Na_2CO_3, was added in order to trinitrophenylate the bovine serum albumin in the conjugate. The reaction mixture was stirred for 6 hr in the dark at 37° and then centrifuged and washed six times with 200 ml of phosphate-buffered saline, stirred for 6 hr at room temperature with 100 ml of 8 M urea, and washed five times with 100 ml of 8 M urea and seven times with 200 ml of phosphate-buffered saline until the optical density at 280 mμ of the supernatant was less than 0.1 and the optical density at 350 mμ, less than 0.05. The TNP-bovine serum albumin–bromoacetylcellulose immunoadsorbent was then treated with 0.1 M 2-mercaptoethanol in 0.02 M Tris-HCl buffer (pH 8.2) (100 ml) for 2 hr at room temperature in order to remove the TNP groups from *O*-TNP-tyrosine and $N_{(Im)}$-TNP-histidine, according to Shaltiel (1967). After centrifugation, the orange supernatant fluid was discarded and the immunoadsorbent was washed several times with phosphate-buffered saline until the optical density at 350 mμ of the washing solutions was less than 0.05. The TNP-bovine serum albumin–bromoacetylcellulose immunoadsorbent was stored at 4° in a moist state.

Isolation of Anti-DNP Antibodies. The TNP-bovine serum albumin–bromoacetylcellulose immunoadsorbent was used for the isolation of anti-DNP antibodies. As was indicated by Little and Eisen (1966), the use of a cross-reacting immunoadsorbent, which competes less well with the homologous hapten used for elution of the antibodies, should lead to a higher yield of the purified antibodies. The antiserum (250 ml) was mixed

with the TNP-bovine serum albumin–bromoacetylcellulose immunoadsorbent and was stirred overnight at 4°. After centrifugation, the immunoadsorbent was washed with phosphate-buffered saline until the optical density at 280 mμ of the washing solutions was less than 0.1. The immunoadsorbent was then suspended in 50 ml of 0.05 M DNP-OH in phosphate-buffered saline, adjusted to pH 7.3, and incubated for 1 hr at 37° with stirring. After centrifugation, the yellow supernatant fluid containing the eluted antibodies and dinitrophenol was passed through a double-layered DEAE-cellulose Dowex 1 (1.8 × 6 cm each) column equilibrated and developed with phosphate-buffered saline, as was suggested by Little and Eisen (1966). The purified antibodies eluted from the column were obtained in a yield of 60–75% of the antibodies present in the serum. The antibody solution was dialyzed against phosphate-buffered saline at 4° for 48 hr and concentrated by vacuum dialysis to a concentration of 20 mg/ml.

Preparation of Rabbit IgG. This was prepared from pooled rabbit sera by precipitation with 37% saturated $(NH_4)_2SO_4$ at 4° followed by chromatography on DEAE-cellulose in 0.0175 M phosphate buffer (pH 6.3) (Levy and Sober, 1960). Fluorescein–IgG was prepared according to McKinney *et al.* (1964). The Fc fragment of rabbit IgG was prepared from a papain digest (Porter, 1959) of the IgG by repeated recrystallizations.

*Preparation of Poly-*DL*-alanyl Mildly Reduced and Alkylated Rabbit IgG.* The procedure used for poly-DL-alanylation was that described by Fuchs and Sela (1965) using a 1:1 weight ratio of *N*-carboxy-DL-alanine anhydride to IgG. However, it was decided to perform the polyalanylation on the antibodies (or IgG) after a mild reduction and alkylation which is sufficient to open the interchain disulfide bridges in IgG (Fleischman *et al.*, 1962).

*Separation of Polypeptide Chains of Poly-*DL*-alanyl IgG and Poly-*DL*-alanyl Antibody.* Mild reduction and alkylation of IgG or anti-DNP antibodies were performed according to Fleischman *et al.* (1962), except that the concentration of 2-mercaptoethanol was 0.1 M in 0.2 M Tris-HCl buffer (pH 8.2). After alkylation with iodoacetamide (10% molar excess over the 2-mercaptoethanol) the solution was dialyzed against 0.05 M sodium phosphate buffer (pH 7.0) for 24 hr. The solutions of the mildly reduced and alkylated IgG or $_n$ antibodies were then used for polyalanylation as mentioned above. The mildly reduced and alkylated (Ala)-$_n$IgG or $(Ala)_n$-antibody solutions were dialyzed against phosphate-buffered saline and were concentrated by vacuum dialysis. To separate the polypeptide chains, the solution was applied to a Sephadex G-100 column (2.5 × 150 cm) equilibrated with 1 M propionic acid (Fleischman *et al.*, 1963). Only the nonaggregated $(Ala)_n$ heavy chains were pooled, dialyzed against water, and freeze dried. The $(Ala)_n$ light chains were similarly treated.

*Preparation of Poly-*DL*-alanyl Fd Fragment.* This was prepared by the cleavage of $(Ala)_n$ heavy chain with CNBr, essentially according to the procedures used by Cahnmann *et al.* (1966) for the cleavage of rabbit IgG, except that the cleavage was performed in 0.05 N HCl–0.14 M CNBr for 2 hr at room temperature. The $(Ala)_n$ Fd fragment was isolated by chromatography on a Sephadex G-75 column equilibrated with phosphate-buffered saline as described in the Results section. The CNBr–Fd fragment, denoted C-1 (Givol and Porter, 1965), used as a control in starch gel electrophoresis, was prepared by the cleavage of heavy chains with CNBr in 70% formic acid.

Complete Reduction of $(Ala)_n$ Heavy Chain and $(Ala)_n$-Fd Fragment. The freeze-dried $(Ala)_n$ heavy chain or $(Ala)_n$-Fd was dissolved (10 mg/ml) in 8 M guanidine hydrochloride–0.5 M Tris-HCl buffer (pH 8.2); 2-mercaptoethanol was added to a final concentration of 0.4 M and the closed vessels containing the reaction mixture were incubated for 4 hr at room temperature. At the end of the reduction period, an aliquot (2–3 mg of protein) was withdrawn for alkylation with iodoacetamide (70% molar excess over that of 2-mercaptoethanol) which was dissolved in 8 M guanidine HCl–2 M Tris-HCl (pH 8.2). During the alkylation reaction, the pH was kept at 8.2 by the addition of 2 M Tris. The carboxamidomethylated protein solutions were dialyzed exhaustively against distilled water, hydrolyzed in 6 N HCl, and subjected to amino acid analysis in order to determine the content of *S*-carboxymethylcysteine residues. The rest of the reduced proteins was used for the reoxidation experiment.

Reoxidation of Fully Reduced $(Ala)_n$ Heavy Chain and $(Ala)_n$-Fd Fragment. After 4-hr reduction, the reduced protein solutions (10 mg/ml) were diluted 500-fold with 6 M guanidine hydrochloride–0.5 M Tris-HCl buffer (pH 8.5) precooled to 4°, so that the final protein concentration was 20 μg/ml. The solutions were dialyzed against 12 l. of 0.1 M Tris-HCl buffer–0.001 M EDTA (pH 8.5) at 4°. The buffer solution was changed every 12 hr over a period of 60 hr, after which the protein solutions were dialyzed against phosphate-buffered saline for 36 hr. The dilute protein solutions were then concentrated by vacuum dialysis to a concentration of 2–3 mg/ml. The concentrated solutions contained some precipitate (accounting for 15% of the protein in the $(Ala)_n$ heavy-chain solution and about 10% of the protein in the $(Ala)_n$-Fd solution), which was centrifuged and discarded. The clear solutions were used for the assay of the binding activity of the regenerated $(Ala)_n$ heavy chain and $(Ala)_n$-Fd fragment.

Antibody Binding by Equilibrium Dialysis. Binding measurements were performed with α-*N*-[^{3}H]acetyl-ε-DNP-L-lysine as hapten. This was prepared by the reaction of ε-*N*-DNP-L-lysine with [^{3}H]acetic anhydride essentially according to an adaptation of the method described by Fraenkel-Conrat (1957) for the acetylation of proteins, developed by J. R. Little (personal communication, 1967). ε-DNP-L-lysine (10 μmoles) was dissolved in 10 ml of water and mixed with 10 ml of saturated sodium acetate solution. The solution was cooled to 0° and small aliquots (5–10 μl) of [^{3}H]acetic anhydride (25.5 mg/0.1 ml of benzene) were added at 10-min intervals until 200 mCi was added in a period of 1 hr. The reaction mixture was incubated overnight at 0°. The solution was brought to a temperature of 20° and was acidified by the addition of 5 ml of concentrated HCl. The α-*N*-[^{3}H]acetyl-ε-DNP-L-lysine was extracted twice with 35 ml of ether and three more times with 10 ml of

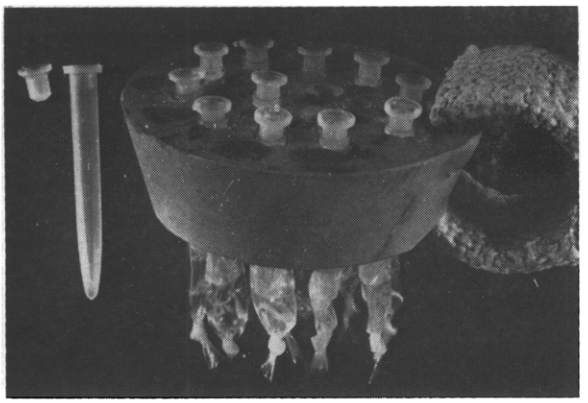

FIGURE 1: Equilibrium dialysis assembly. Right: The rubber stopper holding the dialysis bags. Left: The Beckman microfuge tube used for the preparation of the polyethylene tube, the ring, and the cap for the dialysis bags (see Experimental Section for details).

ether. The ether extracts were pooled and evaporated to dryness. The oily product was dissolved in 4.5 ml of 95% ethanol and applied to a DEAE-cellulose column (0.9 × 25 cm) equilibrated with 0.01 M acetic acid. The column was developed with 300 ml of 0.01 M acetic acid, after which the yellow α-*N*-[^{3}H]acetyl-ϵ-DNP-L-lysine was eluted as a sharp band with 0.01 N HCl. The product was shown to be chromatographically pure and it was obtained in 71% yield. Its absorption spectrum was identical with that of ϵ-DNP-L-lysine in the range of 270–400 mμ. The specific activity was 23,500 cpm/mμmole.

The apparatus used for the equilibrium dialysis studies (Figure 1) was designed to allow simultaneous measurements of many different preparations with multiple sampling during the period of measurement, thus permitting the comparison of binding data obtained under identical conditions. A large rubber stopper (7-cm diameter, 2.5 cm width) was used to hold the dialysis bags; 10–20 holes (4-mm diameter) were cut through it and a 12 cm long, washed Visking $^8/_{32}$ dialysis tubing was passed through each hole. Tapered polyethylene tubes (3.3 cm long) (prepared by cutting the bottom tip and the top rim from the Beckman microfuge centrifuge tube) were inserted through the dialysis casings from the upper side of the stopper, so as to hold them open at the top end, and fasten them to the rubber stopper. The dialysis casings were further fastened to the rubber stopper and to the polyethylene tube, by adjusting the cut-off top rim ring of the centrifuge tube around the dialysis bag (from the bottom side of the rubber stopper) and pressing it up toward the bottom of the rubber stopper. The casings were then tied 2 cm below the lower end of the polyethylene tube and the casings were expanded by air pressure. The expanded part of the bag (below the polyethylene tube) could contain a volume of 0.5 ml and the bottom end of the bag could be reached through the open top by a 20-μl microcaps disposable pipet (Drummond Scientific Co., Broomal, Pa.). The caps of the polyethylene centrifuge tubes were used to cover the samples during dialysis, and one hole in the rubber stopper was left free for air equilibration. Dialysis was performed by immersing the bags in a 200-ml jar having an opening which fits the size of the rubber stopper. Protein solutions inside the dialysis bags were equilibrated at 7° with magnetic stirring against a 200-ml solution of 0.15 M NaCl–0.02 M sodium phosphate buffer (pH 7.2), containing the hapten α-*N*-[^{3}H]acetyl-ϵ-DNP-L-lysine at concentrations ranging from 1×10^{-9} to 2×10^{-5} M, and a drop of toluene. One bag was used as a control for buffer alone to measure equilibration of the hapten. Dialysis of 48 hr was found to be necessary to reach equilibrium except in the lower hapten concentration where 72 hr was required. After 72-hr equilibration against the lowest hapten concentration (1×10^{-9} M), a sample of 20 μl was withdrawn from each of the bags for counting and the "outside" solution was replaced with a solution of higher hapten concentration. After 48 more hr, again a sample of 20 μl was withdrawn for counting and the "outside" solution was replaced. This was repeated six to eight times with increasing hapten concentration solutions. Free hapten concentration was determined by sampling and counting 0.1–0.5 ml of the "outside" solution at the time of each of the above measurements, as compared with the data obtained from the control bag containing only the buffer. The agreement between these two measurements was better than 95% which indicates that the nonspecific casing binding was negligible. Variations in protein concentration inside the dialysis bags during the binding studies were determined by using a fluorescent marker (about 0.05 μg of fluorescein–IgG) which was introduced into each bag, and measuring the fluorescence of a sample (20 μl) from each bag at the time of sampling for counting. The protein concentration inside the dialysis bags ranged from 30 μg/ml for high-affinity material (such as intact antibodies, mildly reduced and alkylated antibodies, and mildly reduced and alkylated $(Ala)_n$ antibodies) to 2–3 mg/ml for low-affinity material (such as $(Ala)_n$ heavy chains or $(Ala)_n$-Fd fragments). Bound hapten concentration was determined by subtracting free hapten concentration from total hapten concentration inside the bags, after making the necessary corrections for variations in protein concentration. The binding data were plotted as $1/r$ *vs.* $1/C$, according to the equation, $1/r = (1/nK_A)((1/C) + (1/n))$ (Eisen, 1964b), where r is moles of hapten bound per mole of antibody at free hapten concentration C, and n is the valency of the antibody preparation. K_A, the average association constant, was graphically determined as the value of $1/C$, when $r = n/2$. The valency, n, was determined as the limiting r value achieved upon extrapolation of $1/r$ to infinite hapten concentration ($1/C = 0$). Thus, the extrapolated $1/r$ value multiplied by 2 corresponds to a value of $1/C$ which equals K_A.

The molecular weights of the different preparations were taken as 150,000, 56,000, and 30,000 for the purified antibodies, $(Ala)_n$ heavy chain, and $(Ala)_n$-Fd fragment, respectively.

Physical Methods. Spectrophotometric measurements were made with a Zeiss PMQII spectrophotometer. The extinction coefficients used ($E^{1\%}_{279}$ at pH 7.3) were 15.5, 15.1, and 15.8 for purified anti-DNP antibodies (Eisen and Siskind, 1964), anti-DNP-$(Ala)_n$ heavy chain, and anti-DNP-$(Ala)_n$-Fd fragment, respectively (as de-

TABLE I: Binding of α-*N*-[^{3}H]Acetyl-ϵ-DNP-L-lysine by Anti-DNP Antibodies.

Sample	K_A ($M^{-1} \times 10^{-8}$)	Hapten Binding Sites/ Mole
Antibodies	2.5	2.0
Mildly reduced and alkylated antibodies	2.5	1.9
Mildly reduced and alkylated $(Ala)_n$ antibodies	0.5	1.8

The procedures for the preparations of the different antibody samples are described in the Experimental Section. K_A is defined in the Experimental Section and was calculated from duplicate equilibrium dialysis measurements at 7°.

termined by micro-Kjeldahl nitrogen analysis). Fluorimetric measurements of fluorescein–IgG were made with an Aminco-Keirs spectrophosphorimeter (absorption, 460 mμ; emission, 520 mμ).

Sedimentation measurements were carried out in a Spinco Model E ultracentrifuge equipped with a schlieren optical system. Samples (3 mg/ml) were sedimented at 56,100 rpm in phosphate-buffered saline.

Starch gel electrophoresis was performed in 8 M urea–formate starch gel (pH 3.4) (Edelman and Poulik, 1961). The gels were run vertically for 18 hr at 5.5 V/cm and at room temperature.

Immunological Methods. Antisera to rabbit IgG and to rabbit light chain were prepared by intramuscular injections of goats with the antigens in complete Freund's adjuvant.

Double immunodiffusion analyses were performed according to Ouchterlony (1953).

Amino Acid Analyses. Hydrolysis was performed in constant-boiling HCl in sealed evacuated tubes at 110° for 22 hr. A Beckman Model 120B amino acid analyzer, equipped with 6.3 V "EEL" Model $^{106}/_{20}$ high-sensitivity unit, was used for the amino acid analyses (Spackman *et al.*, 1958).

The enrichment in alanine residues by the polyalanylation and the number of *S*-carboxymethylcysteine residues formed during the alkylation were computed assuming 33 residue moles of aspartic acid and 30 residue moles of leucine per mole of heavy chain (Fleischman *et al.*, 1963), and 17 residue moles of leucine/mole of Fd fragment (Cebra *et al.*, 1968). Homoserine was determined on the long column of the analyzer in hydrolysates that were treated with pyridine–acetate buffer (pH 6.5) for 1 hr at 100° prior to the analysis (Ambler, 1965).

Radioactivity Measurements. Samples were counted in 10 ml of Bray's (1960) solution in a Packard Tri-Carb liquid scintillation spectrometer.

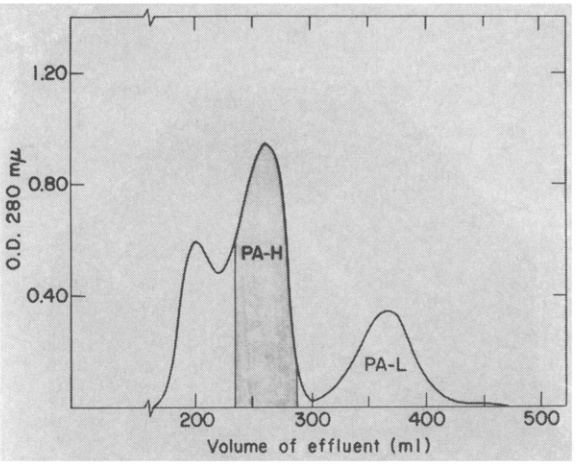

FIGURE 2: Elution pattern of mildly reduced and alkylated anti-DNP-$(Ala)_n$ antibodies (105 mg) from a Sephadex G-100 column (2.5 × 150 cm) equilibrated and developed in 1 M propionic acid at 4°. The material under the hatched area was pooled and used as $(Ala)_n$ heavy chain throughout the study.

Results

Antibody Preparation. The use of a cross-reacting immunoadsorbent, TNP-bovine serum albumin–bromoacetylcellulose, for the absorption of anti-DNP antibodies and the elution with the homologous hapten, dinitrophenol, yielded high recovery of purified antibody, amounting to 60–75% of the antibodies present in the antiserum. The removal of the DNP from the eluted antibody solution could be achieved by passage through a Dowex 1 column. However, it was found necessary to pass the eluted antibody solution first through a DEAE-cellulose column, to remove trace amounts of antigen (TNP-bovine serum albumin), which were probably released from the immunoadsorbent during the isolation procedure. Upon sedimentation in the ultracentrifuge the isolated antibody gave one symmetrical peak of 6.5 S, free of any 19S component. The absence of contaminating hapten was demonstrated by measuring the optical density of the antibody solution at 350 mμ, which was less than 1% of that at 279 mμ, and the purity of the antibody was established by equilibrium dialysis which showed that 2.0 moles of the hapten, α-*N*-[^{3}H]acetyl-ϵ-DNP-L-lysine, were needed for the complete saturation of 1.0 mole of antibody. The average association constant, K_A, of this preparation was 2.5 × 10^8 M^{-1} (Table I). The mild reduction and alkylation did not affect at all the affinity of the antibodies, whereas poly-DL-alanylation caused a fivefold drop in the association constant. Nevertheless, no significant loss in the number of binding sites of the antibodies was observed (Table I).

Poly-DL-alanyl Heavy Chain. The poly-DL-alanylated heavy and light chains of $(Ala)_n$ mildly reduced and alkylated anti-DNP antibodies were separated on a Sephadex G-100 column in 1 M propionic acid. The elution pattern is depicted in Figure 2. The recovery of light chain was 25% of the total absorbancy at 280 mμ. The pooled fractions of heavy chain (hatched area in Figure 2) were checked for contamination with light chain, by

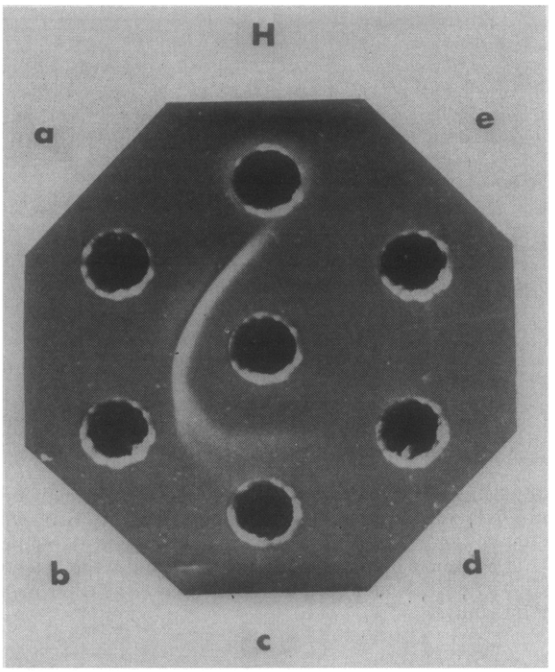

FIGURE 3: Double immunodiffusion pattern of goat antiserum to rabbit light chain (center well) against $(Ala)_n$ heavy chain (8 mg/ml) (H) and $(Ala)_n$ light chain: 0.5 mg/ml (a), 0.2 mg/ml (b), 0.1 mg/ml (c), 0.05 mg/ml (d), and 0.008 mg/ml (e). Diffusion of the reactants was carried out for 48 hr at room temperature.

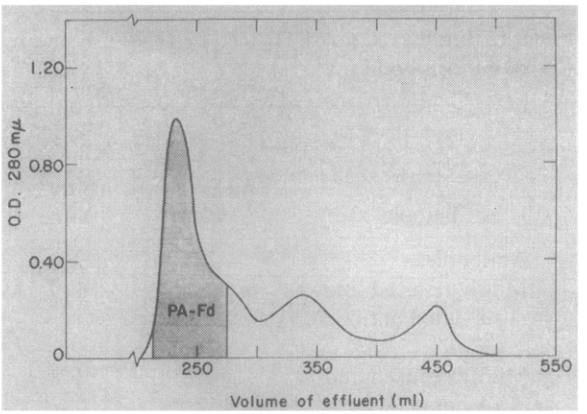

FIGURE 4: Elution pattern of a CNBr digest of anti-DNP-$(Ala)_n$ heavy chain (80 mg) from a Sephadex G-75 column (2.5 × 140 cm) equilibrated and developed in phosphate-buffered saline at room temperature. The CNBr digest was dialysed against phosphate-buffered saline prior to the application to the column and the small amount of precipitate formed during dialysis was discarded. The material under the hatched area was pooled and used as $(Ala)_n$-Fd fragment.

double immunodiffusion against goat anti-light chain, using decreasing concentrations of $(Ala)_n$ light chain for comparison. As can be seen in Figure 3, the heavy chain contained less than 1.25% (w/w) light chain. The lyophilized $(Ala)_n$ heavy chain was completely soluble at neutral pH (phosphate-buffered saline) at a concentration of 20 mg/ml. The enrichment in alanine residues was found to be 83 moles of alanine per mole of heavy chain. The sedimentation coefficients, $s_{20,w}$, of the isolated $(Ala)_n$ heavy chain was 4.8 S with a minor component of 2.5 S. The affinity of the isolated $(Ala)_n$ heavy chains was 1% of that of the intact antibody, whereas the number of combining sites per $(Ala)_n$ heavy chain that could be determined by equilibrium dialysis was 0.26. This figure was taken as 100% for the evaluation of the recovered binding sites after reduction and reoxidation. The $(Ala)_n$ light chain showed no binding under these conditions of measurement.

Poly-DL-*alanyl Fd Fragment*. The isolation of an active $(Ala)_n$-Fd fragment (which is the N-terminal half of the heavy chain) was achieved by a mild cleavage of $(Ala)_n$ heavy chain with CNBr; under the conditions used, 30–35% of the methionine residues were converted to homoserine. $(Ala)_n$ heavy chain (66 mg) was dissolved in water (5 ml) and a freshly prepared solution of 0.1 N HCl–0.28 M CNBr (5 ml) was added to it. The reaction mixture was incubated at 23° for 2 hr, and then dialyzed against phosphate-buffered saline at 4° for 48 hr. Some precipitate that was formed during dialysis was centrifuged and discarded, and the supernatant fluid, containing 82% of the total absorbancy at 280 mμ, was applied to a Sephadex G-75 column equilibrated and developed with phosphate-buffered saline (Figure 4).

Starch gel electrophoresis was used to identify the materials emerging from the column under the various peaks, as compared with the CNBr–Fd fragment, denoted C-1 (Givol and Porter, 1965), obtained after cleavage of the heavy chain with CNBr under more drastic conditions (70% formic acid). The material under the first peak, containing 55% of the total absorbancy at 280 mμ of the starting material, was shown to be similar to the C-1 fragment (Figure 5), but slightly contaminated by faster moving components. The material under the other peaks, including the precipitate formed during dialysis of the CNBr digest, migrated on starch gel electrophoresis similarly to fragments derived from the Fc portion of the heavy chain (Givol and Porter, 1965). The material under the hatched area in Figure 4, denoted $(Ala)_n$-Fd, was lyophilized and used for further experiments. The $(Ala)_n$ Fd fragment was completely soluble in phosphate-buffered saline at a concentration of 10 mg/ml and the enrichment in alanine was found to be 49 moles of alanine residues/mole of $(Ala)_n$-Fd fragment. Otherwise, the amino acid analysis of this fragment was very similar to that of C-1 (Cebra *et al.*, 1968).

Upon sedimentation in the ultracentrifuge, $(Ala)_n$-Fd fragment gave one component with $s_{20,w}$ = 2.7 S. The antigenic characterization of $(Ala)_n$-Fd is shown in Figure 6. It is apparent that the $(Ala)_n$-Fd fragment contains common determinants with $(Ala)_n$ heavy chain, but lacks any antigenic determinants common with Fc. On the basis of the yield of $(Ala)_n$-Fd, its amino acid analysis, starch gel electrophoretic pattern, and antigenic characterization, it is concluded that $(Ala)_n$-Fd represents a genuine fragment and not undigested heavy chain. The binding activity of anti-DNP-$(Ala)_n$-Fd fragment was measured by equilibrium dialysis. It had the same association constant, K_A, as the parent $(Ala)_n$ heavy chain, and 0.16 of a combining site/fragment (Table II).

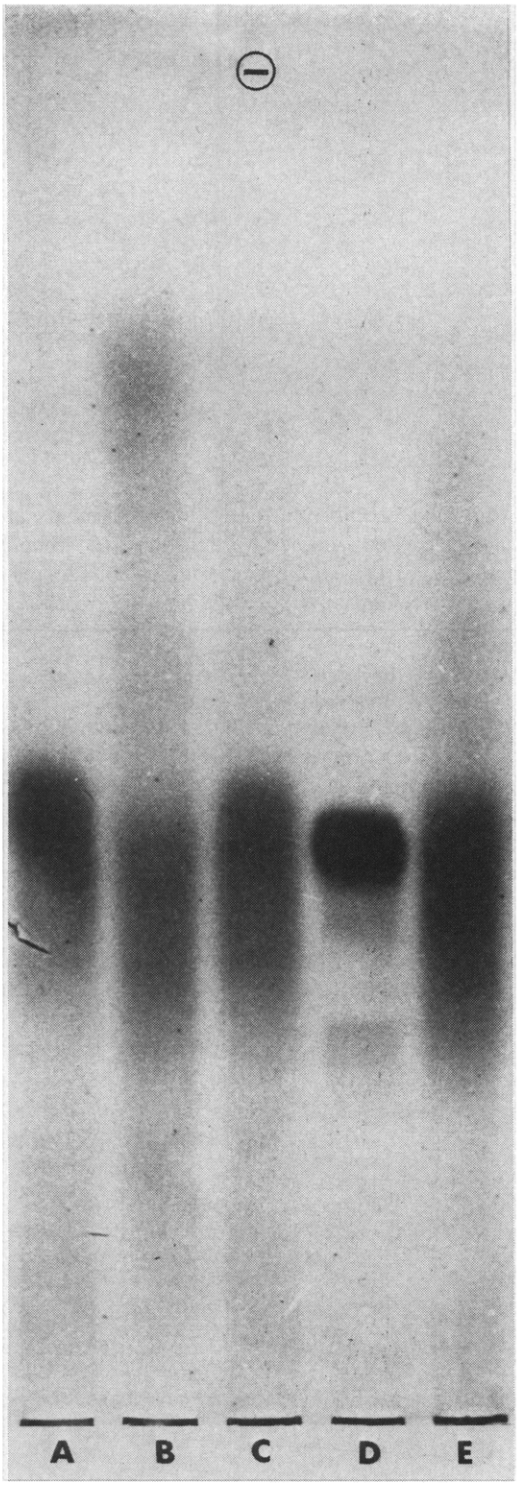

FIGURE 5: Starch gel electrophoresis pattern of CNBr-treated $(Ala)_n$ heavy chain. (A) C-1 obtained after digestion of rabbit IgG heavy chain with CNBr in 70% formic acid (Givol and Porter, 1965). (B) CNBr digest of anti-DNP-$(Ala)_n$ heavy chain after dialysis against phosphate-buffered saline. (C) Anti-DNP-$(Ala)_n$-Fd fragment (see Figure 4). (D) Anti-DNP-$(Ala)_n$ heavy chain (see Figure 2). (E) $(Ala)_n$-Fd fragment from normal rabbit IgG $(Ala)_n$ heavy chain. The electrophoresis was performed at 5.5 V/cm, 40 mA for 18 hr at room temperature and at pH 3.4.

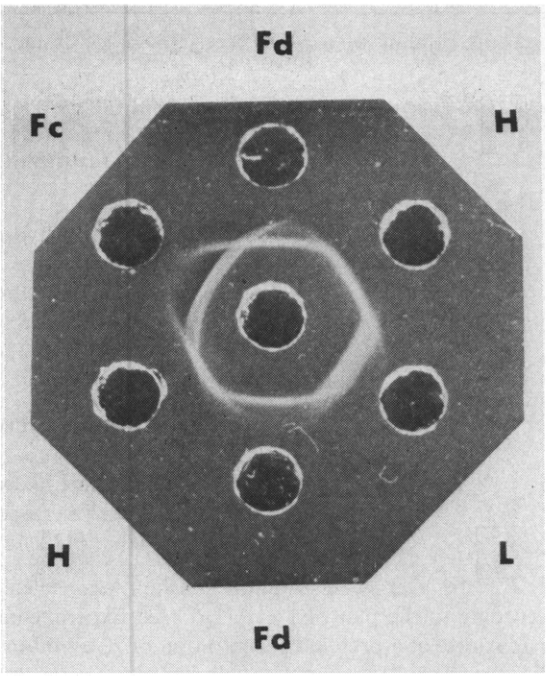

FIGURE 6: Double immunodiffusion pattern of rabbit IgG fragments. The center well contained a mixture of goat antiserum to rabbit IgG and goat antiserum to rabbit light chain. (Fd), $(Ala)_n$-Fd fragment; (Fc), Fc fragment from normal IgG; (H), $(Ala)_n$ heavy chain; and (L), $(Ala)_n$ light chain. Protein concentrations were 0.1% for $(Ala)_n$ light chain and 0.2% for the others.

*Full Reduction and Reoxidation of Poly-*DL*-alanyl Heavy Chain and Poly-*DL*-alanyl Fd Fragment.* The extent of reduction of anti-DNP $(Ala)_n$ heavy chain and anti-DNP $(Ala)_n$-Fd fragment in 8 M guanidine hydrochloride (pH 8.2) in the presence of 0.4 M 2-mercaptoethanol was determined by the amount of *S*-carboxymethylcysteine present in the alkylated samples (see Experimental Section). $(Ala)_n$ heavy chain contained 13.0 residues of *S*-carboxymethylcysteine/molecule and $(Ala)_n$-Fd fragment, 6.4 residues of *S*-carboxymethylcysteine/molecule. In both preparations, no trace of half-cystine could be detected. This is in agreement with the reported *S*-carboxymethylcysteine content of heavy chain (Fleischman *et al.*, 1963) and of C-1 (Cebra *et al.*, 1968).

The samples that were allowed to reoxidize at pH 8.5 at a concentration of 20 μg/ml were concentrated by vacuum dialysis and were recovered in a yield of 85 and 90% for $(Ala)_n$ heavy chain and $(Ala)_n$-Fd fragment, respectively.

The reoxidized materials had the same sedimentation coefficients as the untreated materials. Antigenic analysis of the reoxidized materials by double immunodiffusion is shown in Figure 7. It can be seen that the reoxidized $(Ala)_n$ heavy chain and $(Ala)_n$-Fd fragment give lines of complete identity with that of the untreated parent materials. The spurs of $(Ala)_n$ heavy chain over the precipitin line of $(Ala)_n$-Fd fragment are clear both for the untreated and the reoxidized materials. It can be concluded that the antigenic determinants were regained upon reoxidation of the reduced materials.

TABLE II: Binding of α-*N*-[^{3}H]Acetyl-ϵ-DNP-L-lysine, by Anti-DNP-$(Ala)_n$ Heavy Chains and $(Ala)_n$-Fd Fragments.[a]

Sample	Treatment (M)[b]	K_A($M^{-1} \times 10^{-6}$)	Hapten Binding Sites	Recovd Binding Sites (%)[c]
$(Ala)_n$-Heavy chains		2.4	0.260	100
	Guanidine hydrochloride (8)	1.6	0.140	54
	Guanidine hydrochloride (8)–2-mercapto-ethanol (0.4)	1.3	0.096	37
$(Ala)_n$-Fd fragments		2.4	0.160	100
	Guanidine hydrochloride (8)	0.75	0.120	75
	Guanidine hydrochloride (8)–2-mercapto-ethanol (0.4)	0.6	0.093	59

[a] K_A, the average association constant, was calculated from duplicate equilibrium dialysis measurements at 7°, according to the plot of $1/r$ *vs.* $1/C$ (see Experimental Section). [b] The treated samples were allowed to "renature" or reoxidize at a protein concentration of 20 μg/ml by dialysis against 0.1 M Tris-HCl buffer–0.001 M EDTA (pH 8.5) at 4° for 60 hr. [c] The number of binding sites for the untreated samples was taken as 100%.

Recovery of Binding Sites and Affinity upon Reoxidation of Fully Reduced Poly-DL-*alanyl Heavy Chain and Poly*-DL-*alanyl Fd Fragment.* The recovery of antibody activity following reoxidation after complete reduction was determined by equilibrium dialysis. In order to evaluate denaturing effects of guanidine hydrochloride alone, samples were also incubated in 8 M guanidine hydrochloride–0.5 M Tris-HCl (pH 8.2) for 4 hr without 2-mercaptoethanol and were allowed to "renature" under the same conditions as for the reduced samples. The binding curves for the untreated, the reoxidized, and "renatured" anti-DNP-$(Ala)_n$ heavy chains are shown in Figure 8, and those for the untreated, the reoxidized and the "renatured" anti-DNP-$(Ala)_n$-Fd fragments are shown in Figure 9. The analogous samples of untreated or reoxidized $(Ala)_n$ heavy chains and $(Ala)_n$-Fd frag-

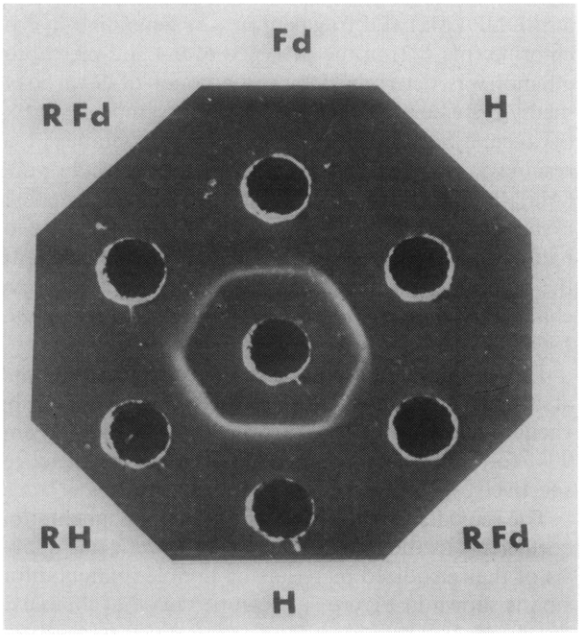

FIGURE 7: Double immunodiffusion pattern of native and reoxidized anti-DNP fragments. The center well contained goat antiserum to rabbit IgG. (Fd), $(Ala)_n$-Fd fragment; (RFd), reoxidized $(Ala)_n$-Fd fragment after reduction in 8 M guanidine hydrochloride; (H), $(Ala)_n$ heavy chain; and (RH), reoxidized $(Ala)_n$ heavy chain after reduction in 8 M guanidine hydrochloride. Protein concentrations were 0.2%.

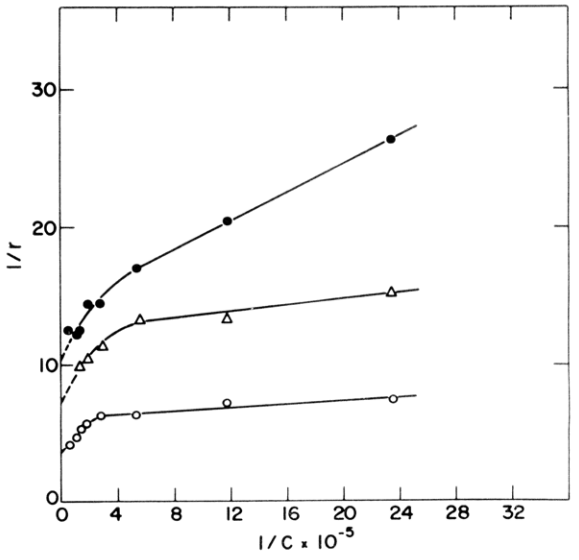

FIGURE 8: Binding curves of anti-DNP-$(Ala)_n$ heavy-chain preparations. r is moles of hapten bound per mole of protein. C is the free hapten concentration. $(Ala)_n$ heavy chain (○). Guanidine hydrochloride treated $(Ala)_n$ heavy chain (△). Fully reduced and reoxidized $(Ala)_n$ heavy chain (●).

TABLE III: Recovery of Specific Activity upon Reoxidation of Fully Reduced Antibody Preparations.

Antibody Preparation	Disulfide Bonds Cleaved	Recov of Soluble Protein (%)	Recov of Act. (%) Based on Amount of Soluble Protein	Recov of Act. (%) Based on Total Amount of Protein	Drop in Assocn Constant	Methods of Measurement	References
Anti-RNase Fab I	6	53	27	14.3	Not determined	Antigen-binding capacity	Haber (1964)
Anti-DNP Fab I	6	30	24	7.1	Not determined	Fluorescence quenching	Whitney and Tanford (1965)
Anti-DNP Fab II	6	43	20	8.4	Not determined	Fluorescence quenching	Whitney and Tanford (1965)
Anti-bovine serum albumin $(Ala)_n$-IgG	23	>95	33	23.0	Not determined	Antigen-binding capacity	Freedman and Sela (1966b)
Anti-DNP-$(Ala)_n$ heavy chain	4–5	85	37	31.5	Twofold	Equilibrium dialysis	This paper
Anti-DNP-$(Ala)_n$-Fd	2–3	90	59	53.0	Fourfold	Equilibrium dialysis	This paper

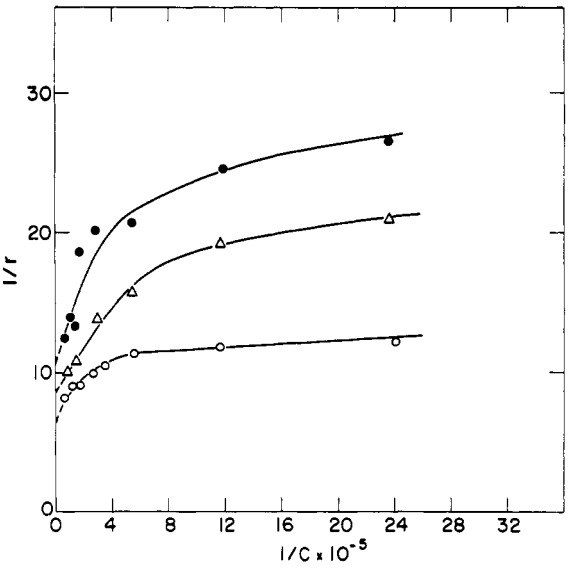

FIGURE 9: Binding curves of anti-DNP-$(Ala)_n$-Fd fragment preparations. $(Ala)_n$-Fd fragment (○). Guanidine hydrochloride treated $(Ala)_n$-Fd fragment (△). Fully reduced and reoxidized $(Ala)_n$-Fd fragment (●).

ments derived from normal IgG did not show any binding of the hapten in the range of free hapten concentration from 4.2×10^{-7} to 5.6×10^{-6} M. Small nonspecific binding did occur at higher free hapten concentration (7.5×10^{-6} to 2×10^{-5} M), amounting to about 5–12% of the binding of the anti-DNP samples, and the binding data of the anti-DNP samples in this range were corrected accordingly. The limiting r values, *i.e.*, moles of hapten bound per mole of fragment at saturation, were determined by extrapolation to infinite free hapten concentration, C. The values thus obtained of the number of hapten binding sites in the untreated, the reoxidized, and the "renatured" samples are given in Table II. It is shown that recoveries of binding sites upon reoxidation of the fully reduced samples were 37.5 and 59% for anti-DNP-$(Ala)_n$ heavy chain and anti-DNP-$(Ala)_n$-Fd fragment, respectively. If the antibody activity found in the reoxidized preparation would be due to a small proportion of unreduced antibody molecules, it would be expected that the same activity would be found in the fully reduced and carboxymethylated anti-DNP-$(Ala)_n$ heavy chain. This experiment was performed and the binding by fully reduced and carboxamidomethylated anti-DNP $(Ala)_n$ heavy chain was identical with that of a control, namely, a $(Ala)_n$ heavy chain derived from normal immunoglobulin G.

It is noteworthy that the reoxidized samples have recovered an association constant, K_A, which is of the same order of magnitude (around 1×10^6 M^{-1}) as that of the untreated samples. This is especially remarkable in view of the 100-fold drop of the association constant between the antibody and the isolated heavy chain.

Discussion

The results of the present investigation confirm previous findings on the recovery of antibody activity following complete reduction and unfolding (Haber, 1964;

Whitney and Tanford, 1965; Freedman and Sela, 1966b), and extend them to include the recovery of specific binding activity of the heavy chain and the Fd fragment. Table III gives a comparison of the main results of previous studies and of the present one. It is apparent that the recoveries of re-formed antibody combining sites greatly exceed the recoveries expected on the basis of a purely random re-formation of disulfide bonds (Sela and Lifson, 1959; Kauzmann, 1959).

The problem of the insolubility of the heavy chain and of the Fd fragment in neutral aqueous buffer was overcome by poly-DL-alanylation (Fuchs and Sela, 1965), which did not decrease significantly the affinity of the antibody (Table I), as was first shown by Karush and Sela (1967). Thus, it became possible to perform the reoxidation of fully reduced $(Ala)_n$ heavy chain or $(Ala)_n$-Fd fragment in the absence of light chain.

The number of hapten binding sites in the native $(Ala)_n$ heavy chain (0.26/chain) is in agreement with previous findings on the activity of heavy chain (Weir and Porter, 1966; Haber and Richards, 1966), and this value was taken as 100% in evaluating the regained activity after reoxidation. It is possible that sites with a very low affinity escaped detection by the equilibrium dialysis technique. The number of 0.26 site/average heavy chain is not due to a slight contamination with light chains, as apparent both from the low affinity of the antibody heavy chain (1% of that of the intact antibody) and from almost complete lack of cross-reaction with goat anti-light chain antibodies (the $(Ala)_n$ heavy chain could be contaminated with less than 1.25% of $(Ala)_n$ light chain; see Figure 3).

The persistence of some low affinity in an Fd fragment isolated from an Fab fraction of an antibody has been described by Roholt *et al.* (1966). In this study, we found that the removal of the C-terminal moiety of antibody $(Ala)_n$ heavy chain by means of mild cleavage with CNBr resulted in a $(Ala)_n$-Fd fragment possessing the same average association constant as the $(Ala)_n$ heavy chain from which it was derived. On the other hand, its limiting r value, 0.16, was significantly lower than the value of 0.26 found for the parent $(Ala)_n$ heavy chain (Table II). Thus, the possibility of measurement of hapten binding by Fd fragment provides direct evidence that it carries the antibody combining site.

This investigation was designed to determine whether the region of the heavy chain having antigen-binding capacity was able to regain this capacity after complete reduction and unfolding, followed by reoxidation in the absence of the light chain. It was further attempted to elucidate whether the Fd region of the heavy chain can regain its specificity after complete reduction and unfolding, upon reoxidation in the absence of both Fc and light chain. For this purpose, both the number and the affinity of the binding sites were measured before and after the total reduction and reoxidation. Freedman and Sela (1966b) already indicated that the recovery of activity by an antibody whose heavy and light chains were separately reoxidized and then mixed together was the same as that of a reoxidized antibody whose chains were not separated.

The present study also shows that all the types of antigenic determinants on the heavy chain and the Fd fragment were re-formed after total reduction and reoxidation of the respective poly-DL-alanylated derivatives (see Figure 7). This is in agreement with previous results reported by Freedman and Sela (1966a).

In the only previous experiments concerned with the affinity of the antibody Fab fragments, after reduction followed by reoxidation, a large decrease in the affinity of the recovered sites was noted (Whitney and Tanford, 1965). These authors suggested as a possible explanation of the poor recovery the fact that the Fab fragment used in these studies consists of two chains and improper recombination of these in the refolding process might account for the poor recovery. If this were so, the recovery of activity of heavy chain should be much greater since it is a single polypeptide chain.

The results of the experiments reported here confirm these expectations. The affinity decrease after complete reduction and unfolding and subsequent refolding and reoxidation is less than twofold for the heavy chain, which is very little considering the great loss of affinity resulting from the preparation of heavy chain and the effect of guanidine hydrochloride itself (Tables I and II). Further, it has been shown that the Fd fragment prepared by mild digestion with CNBr, which retained almost the complete heavy-chain activity, has the capacity to regain almost all of its binding activity in the absence of both light chain and Fc. If the results with the $(Ala)_n$ heavy chain and the $(Ala)_n$-Fd fragment of anti-DNP antibodies, after treatment with 8 M guanidine hydrochloride in the absence of 2-mercaptoethanol, be taken as 100% for the reoxidation experiments, the results given in Table II show that 68.5% of the $(Ala)_n$ heavy chain hapten binding sites and 77.5% of $(Ala)_n$-Fd hapten binding sites, respectively, were recovered.

These results indicate that the information required to form the hapten binding site of the heavy chain is contained in the amino acid sequence of its Fd region. This implies that during antibody biosynthesis the heavy chain may be folded so as to present an antibody binding site prior to association with light chain to form the immunoglobulin molecule.

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