Biochemistry

Volume 11, Number 8 April 11, 1972

Contributions of Heavy and Light Chains of Rabbit Immunoglobulin G to Antibody Activity. I. Binding Studies on Isolated Heavy and Light Chains†

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ABSTRACT: Heavy (H) and light (L) polypeptide chains were prepared from high affinity pooled rabbit anti-2,4-dinitrophenyl (DNP) antibodies by methods previously developed in this laboratory. Their physical properties were similar to those reported previously for the polypeptide chains from nonspecific rabbit immunoglobulin (IgG). In particular, the H chain and the Fd fragment prepared from it were found to be dimeric and to possess only a single binding site for hapten per dimer. As with nonspecific IgG, two classes of L chains were observed, one existing predominantly as monomer and the other as dimer. Average binding constants ($\bar{K}_0$) for ε-[H^3]amino-DNP-L-lysine at 5° were measured by equilibrium dialysis at pH 5.5. The $\bar{K}_0$ observed for mildly reduced and carboxymethylated anti-DNP, H_2, and L chain were 3×10^8 M^{-1}, 6×10^4 M^{-1}, and 200–500 M^{-1}, respectively. The unitary free energies of binding calculated from these data for H_2 and L_2, when summed, account for the free energy of binding by the intact antibody. This result indicates that (1) both chains contribute direct contact residues to the antibody combining site and (2) the active-site regions of the H and L chains are conformationally similar in the intact and isolated states. The fact that the visible spectrum of hapten was perturbed identically by H_2 and intact anti-DNP is further evidence for site integrity and that the tryptophan responsible for the perturbation is located on the H chain. The results are consistent with the hypothesis that a relatively small number of H and L chain genes could generate through random recombination of H and L chains a very large number of functionally discrete immunoglobulin molecules.

Immunoglobulin G[1] is made up of two heavy (H) and two light (L) polypeptide chains held together by both disulfide bonds and noncovalent forces (Edelman, 1959; Edelman and Poulik, 1961; Fleischman *et al.*, 1962). The two chains can be separated by several techniques and the isolated chains "renatured" by their return to dilute buffers at near-neutral pH (Fleischman *et al.*, 1962; Utsumi and Karush, 1964). Amino acid sequences of myeloma heavy and light chains show that both chain types are variable in their amino-terminal regions (Hilschmann and Craig, 1965; Putman *et al.*, 1967; Edelman and Gall, 1969). Along with affinity labeling studies of antihapten antibodies where labeling of both chains is observed (Thorpe and Singer, 1969; Wofsy *et al.*, 1967), the sequence variability in both chains suggests that both contribute in some way to antibody specificity.

Attempts have been made to measure direct binding of antigen by isolated heavy and light chains (Fleischman, 1966; Porter and Weir, 1966; Haber and Richards, 1966; Yoo *et al.*, 1967; Utsumi and Karush, 1964). Although direct binding of antigen by heavy chain is well established, quantitative binding data have been lacking due to the low solubility of heavy chain as well as its tendency to aggregate in dilute buffers. Direct evidence of light chain binding activity has been reported (Yoo *et al.*, 1967; Goodman and Donch, 1965; Mangalo and Raynaud, 1967), but other workers have found no detectable L chain antigen binding activity (Edelman *et al.*, 1963; Utsumi and Karush, 1964).

Conditions under which the heavy chain of normal IgG

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[1] The nomenclature of and the abbreviations for the immunoglobulins and their subunits produced by reduction and proteolysis are those recommended by the World Health Organization (1964).

exists as a stable well-defined dimer of 110,000 molecular weight have recently been described (Björk and Tanford, 1971a; Stevenson and Dorrington, 1970). In this paper we describe the preparation of H and L chains from high affinity rabbit anti-DNP[2] antibody by the procedures developed by these workers. The physicochemical properties of the isolated chains are reported and found to be similar to those of the polypeptide chains of normal rabbit IgG. The major part of the paper consists of a study of the binding of hapten by the individual chains and comparison with binding by the intact antibody.

Materials and Methods

Purification of Anti-DNP. Twenty-two New Zealand white rabbits were immunized with 5 monthly courses of DNP_{29}-B-γ-G in Freund's complete antigen. The first course (5 mg) was given half in the toe pads and half subcutaneously. All subsequent courses (2.5 mg/course) were given subcutaneously. Starting at 2 weeks after the final course, blood was collected at 4-day intervals for 4 additional months. Antisera, obtained after clotting, were clarified by centrifugation at 20,000*g* for 1 hr at 4°, decomplemented by heating at 56° for 20 min, and stored frozen at −20°. Sera from all bleedings were pooled and used for subsequent purification of high affinity anti-DNP antibody. The pooled antisera contained 1.9 mg/ml of anti-DNP antibody precipitable with DNP_{28}-BSA. TNP_{60}-BSA, a heterologous, cross-reacting antigen, precipitated 1.6 mg/ml of anti-DNP antibody, demonstrating the high-average affinity of the pooled antisera (Eisen *et al.*, 1967).

High-affinity anti-DNP antibody was isolated from pooled sera by specific precipitation with TNP_{60}-BSA and subsequent specific dissolution of the immune precipitate with 0.1 M dinitrophenol in 0.15 M NaCl–0.02 M potassium phosphate buffer (pH 7.4). The antigen and dinitrophenol hapten were separated from the antibody by ion-exchange chromatography as described by Eisen *et al.* (1967). Purified antibody was stored under 50% saturated ammonium sulfate at −20° until further use.

Anti-DNP prepared in this manner gave a single precipitin line of IgG mobility by immunoelectrophoresis against goat anti-whole-rabbit serum. Ultracentrifugal analysis of a 4 mg/ml solution showed a major peak (90–95%) with $s_{20,w}$ = 6.5 S and a faster sedimenting component accounting for the remainder. The preparation was 85–90% precipitable with DNP_{28}-BSA and had an absorbance at 360 nm (due to residual hapten) that was less than 1% of the absorbance at 280 nm. Fluorescence quenching titrations of the protein with DNP-lysine showed a maximum quenching of 70% as compared to 72%, reported by Eisen and Siskind (1964), and an affinity constant $\bar{K}_0 > 10^8\ M^{-1}$.

Preparation of Chains and Fd Fragment. Heavy and light chains were prepared from mildly reduced and alkylated antibody as described previously (Björk and Tanford, 1971a). H chains were "renatured" from 1 M propionic acid by dialysis against 0.01 M sodium acetate (pH 5.5). For hapten binding measurements renatured H chains were chromatographed on Sephadex G-200 (Pharmacia Fine Chemicals) in 0.02 M sodium acetate (pH 5.5) without added salt, to minimize aggregation and the subsequent lower yields. H chains were used within 3–4 days after this step since very slow aggregation occurs even at low ionic strength. When necessary, excess material was stored frozen at −20°, but was always rechromatographed on G-200 before use. Since H chain tended to precipitate from solution at room temperature, especially at high ionic strength (~0.1), most measurements were carried out at 5° where molecular aggregation was virtually nonexistent. L monomers and dimers were isolated as described previously (Björk and Tanford, 1971b) by gel chromatography of renatured L chains on Sephadex G-75.

Purified anti-DNP was split into Fab and Fc fragments by limited papain digestion as described by Porter (1959) and modified by Nelson *et al.* (1965). Fc fragment was separated from Fab by crystallization of Fc with dialysis against water. Fab (I) and Fab (II) were then separated by CM-52 (Whatman) chromatography as described by Porter (1959). The Fab (I) and Fab (II) fractions were pooled, mildly reduced with 0.2 M β-mercaptoethanol at 23°, alkylated at 2° with 0.25 M iodoacetamide after 2 hr, and dialyzed exhaustively against distilled water before being used to prepare Fd fragment. The Fd fragment was prepared from Fab fragments essentially as described by Fleischman *et al.* (1963). The fragments were renatured by dialysis against 0.02 M sodium acetate, pH 5.5, as were H and L chains.

Preparation of Radioactive Hapten. [^{3}H]DNP-L-lysine with a high specific activity was prepared from [^{3}H]dinitrofluorobenzene (25.3 Ci/mmole, Amersham-Searle, Chicago), dinitrofluorobenzene (Aldrich, Cedar Knolls, N. J.), and *tert*-Boc-α-NH_2-L-lysine (Pierce, Rockford, Ill.) by a modification of the method of Eisen *et al.* (1968). The labeled product cochromatographed with the authentic compound (Sigma Chemical Co., St. Louis, Mo.) by paper chromatography (2 solvent systems) and had a visible spectrum which was identical with that of the authentic compound. The labeled derivative, in addition, quenched the tryptophan fluorescence of anti-DNP in a manner similar to the authentic compound. The final specific activity was 3.7 Ci/mmole. DNP-lysine concentrations in dilute buffers were determined spectrophotometrically using a molar extinction coefficient of 17,530 at 363 nm (Eisen and Siskind, 1964).

Goat Antisera. Goat antisera to rabbit IgG, Fc, and Fab fragments were prepared as described previously (Björk and Tanford, 1971a). A goat anti-rabbit Fd serum was custom-made by Gateway Antisera Co. (Cahokia, Ill.). This antiserum was adsorbed with rabbit L chain to remove a trace of anti-L activity. The resulting serum reacted strongly with Fd, H chain, and Fab, but not with Fc or L chain as judged by immunodiffusion. A goat antiserum to rabbit L chain was kindly donated by Dr. H. N. Eisen. Immunodiffusion and immunoelectrophoresis were carried out as described previously (Björk and Tanford, 1971a).

Other Reagents. 2,4-Dinitrophenol was purchased from Fisher Chemicals (Pittsburgh, Pa.) and recrystallized from hot, distilled water 3 times before use. Bovine serum albumin was obtained from Armour (Chicago, Ill.) and B-γ-G from Pentex (Kankakee, Ill). DNP and TNP conjugates of the albumin and B-γ-G were prepared and characterized by previously published procedures (Little and Eisen, 1967a) as were fluorescein–IgG conjugates (Coons and Kaplan, 1950). DNP-lysine and DNP-aminocaproic acid were obtained from Sigma (St. Louis, Mo.). Rabbit IgG (Pentex) was lightly iodinated with [I^{125}]NaI (carrier-free; New England Nuclear, Boston, Mass.) using the chloramine-T method (McConahey and

[2] Abbreviations used are: BSA, bovine serum albumin; TNP, 2,4,6-trinitrophenyl; DNP, 2,4-dinitrophenyl; B-γ-G, bovine γ-globulin; anti-DNP, antibody to the DNP hapten; DNP-lysine, ε-amino-2,4-dinitrophenyl-L-lysine; *t*-Boc, *tert*-butoxycarbonyl; DNP-aminocaproate, 2,4-dinitrophenylaminocaproate.

Dixon, 1966). All other reagents and chemicals were reagent grade or better.

Equilibrium Dialysis. Equilibrium dialysis was carried out using modified commercial acrylic cells obtained from Interscience (Baltimore, Md). Experiments with intact antibody and mildly reduced and alkylated antibody were performed in 1-ml cells. In all other experiments, cells with a 0.1-ml capacity were used. Dialysis membranes (Visking dialysis tubing 1-20/64 in. × 0.001 in., Union Carbide, Chicago, Ill.) were treated with a boiling solution of 1% $NaHCO_3$–0.01% EDTA for 20 min followed by exhaustive washing with deionized water and finally washed once with 0.02 M sodium acetate, pH 5.5. The cells were filled with the appropriate protein solution on one side and an equal volume of [^{3}H]DNP-L-lysine on the other, using calibrated syringes. Duplicate cells were used in all experiments. In several experiments, hapten and protein were placed initially on the same side of the membrane with buffer on the other to test for the thermodynamic reversibility of the binding reaction. The cells were sealed with paraffin and rotated at 5–6 rpm in a 5° water bath. Attainment of dialysis equilibrium was determined with controls containing only hapten. Due to cell adsorption of protein at the low protein concentration required (15–30 μg/ml) with intact and mildly reduced and alkylated antibody, these experiments were performed in the presence of 0.1% normal rabbit IgG. Under these conditions adsorption of specific antibody to cell surfaces was found to be negligible. Binding of hapten by normal IgG was negligible under the conditions used. Aliquots (20 or 50 μl) were withdrawn from each side of the 0.1-ml cells with disposable microcaps (Drummond Scientific Co., Broomall, Pa.). Usually 50-μl samples were withdrawn when using 1-ml cells. Larger samples, however (up to 0.5 ml), were taken in those cases where hapten concentrations were less than 10^{-9} M. Samples were counted with a Packard Model 3375 Tri-Carb liquid scintillation spectrometer in 10 ml of Triton X-100–toluene (1:2) scintillation fluid containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter of toluene. Counting error was less than 0.5%. In experiments where the difference in counts per minute between the two sides of the membrane was less than 20%, duplicate aliquots were counted with a precision of 1%. Recovery of radioactivity was better than 90% in all binding experiments. Samples were recounted after the addition of 25 μl of a [^{3}H]toluene standard (Packard Instruments) in order to determine counting efficiency. Only in those cases where protein and hapten concentrations were high (*e.g.*, L chain experiments) or where large aliquots were counted were quenching corrections necessary.

Protein concentrations were measured before and after equilibrium dialysis in the following manner. In L chain experiments, 10-μl samples were withdrawn from the protein-containing side of each cell and diluted into 1.0 ml of buffer. The absorbancy at 280 nm was measured using an identical dilution of an aliquot from the nonprotein side of the cell as the blank. In the case of H chain or Fd fragment, the concentration was checked by dilution of 20-μl samples into 1.0 ml of 10% trichloroacetic acid and measuring the resulting absorbance at 330 nm due to light scattering. Hapten absorbancy was negligible at this wavelength. The observed absorbance was converted to protein concentration from a linear plot of A_{330} *vs.* protein concentration determined with protein solutions of known concentration. In experiments with protein concentrations of 15–30 μg/ml, where measurement of protein concentrations by usual techniques was not feasible, a small amount (~1 μg/ml) of a fluorescein–IgG conjugate was added. The fluorescence (λ_{EX} 480; λ_{EM} 520) was then measured before and after the dialysis experiment. This method was checked using ^{125}I-labeled antibody and was found to give good agreement. In all experiments the variation of the final protein concentrations from the initial values was less than 10%. The initial value was used in subsequent calculations if the variation was less than 5%.

All measurements were corrected for small but significant binding by the appropriate nonspecific rabbit chains. The molar concentrations of protein were calculated using 150,000, 110,000, and 50,000 as the respective molecular weights of IgG, H chain, and Fd fragment.

Binding data were calculated and plotted according to the procedure of Scatchard (1949). Values of n, the number of antibody binding sites, and ($\bar{K}_0$), the average binding constant, were calculated as in Karush (1962). Average free energies of binding ($\Delta\bar{G}_u^\circ$) are given in unitary units to avoid inclusion of the cratic entropy term common to all ligand binding reactions (Karush, 1962).

Other Methods. Fluorescence quenching was performed as described by Velick *et al.* (1960). Excitation was at 290 nm and the emission measured at 345 nm. Maximum quenching (Q_{max}) was determined by the method of Eisen and McGuigan (1971). Results were expressed in terms of fluorescence relative to the initial fluorescence after correction for dilution by hapten. All data were corrected for nonspecific quenching due to attenuation of the excitation beam by free hapten absorption. This was determined by identical titrations of *N*-acetyltryptophan (1×10^{-5} M) as described by Day *et al.* (1963). Identical results were obtained using either an Aminco-Bowman or a Turner spectrofluorometer.

Optical rotatory dispersion (ORD) spectra were determined with a Cary Model 60 recording spectropolarimeter. Cell path length and protein concentration were chosen so that the total absorbancy was always less than 2 over the region of interest (220–260 nm).

Difference spectra between bound hapten and free hapten were determined using a Cary Model 14 spectrophotometer equipped with water-jacketed cuvet holders. Tandem cell holders were used in each compartment with a matched set of four 1-cm microcuvets. The absolute absorption spectrum for bound hapten was determined by dialyzing proteins against hapten and measuring the resulting spectrum using the dialysate in the reference compartment.

Sedimentation velocity experiments were performed using a Beckman Model E analytical ultracentrifuge equipped with both schlieren and interference optics. In some experiments, a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner and multiplexer accessory as well as an electronic speed control was used. All sedimentation velocity measurements were performed at 60,000 rpm at 23–25° and the measured s value was corrected to $s_{20,w}$ by the standard procedure of Svedberg and Pedersen (1940).

Molecular weights were determined by sedimentation equilibrium using the high-speed meniscus depletion technique when fringe optics were used (Yphantis, 1964). Both the conventional low-speed and meniscus depletion techniques were used with scanner optics. With low protein concentrations the method of Chervenka (1969) was used in order to avoid convection due to thermal fluctuations (Yphantis, 1964). All proteins were dialyzed for at least 16 hr if dilute buffer was the solvent. Exhaustive dialysis was not necessary when the photoelectric scanner was used to measure the concentration gradients since buffer absorbancy at 280 nm was negli-

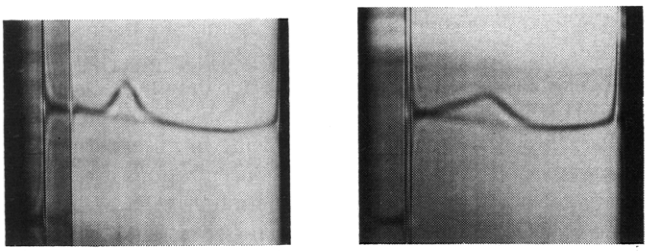

FIGURE 1: Sedimentation velocity patterns of rabbit anti-DNP chains. (a, left) Heavy-chain dimer at 3.0 mg of protein/ml in 0.02 M sodium acetate (pH 5.5)–0.1 M NaCl, 23°; 60,000 rpm, bar angle 50°; picture taken 30 min after top speed. (b, right) Fd fragment at 3.0 mg of protein/ml in 0.02 M sodium acetate (pH 5.5)–0.1 M NaCl, 25°; 60,000 rpm, bar angle 45°; picture taken 72 min after reaching top speed.

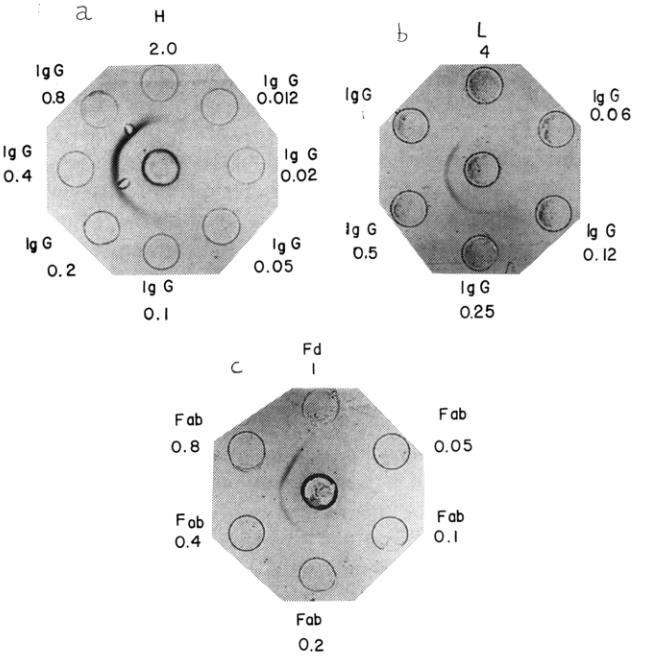

FIGURE 2: Immunodiffusion analysis of isolated anti-DNP chains and fragments. (a) Heavy chain (H) and intact antibody (IgG) *vs.* goat anti-rabbit L-chain serum. (b) Light chain (L) and intact antibody (IgG) *vs.* goat anti-rabbit Fc fragment. (c) Fd fragment and Fab fragment *vs.* goat anti-rabbit L-chain serum. All antisera are in the center wells. The numbers refer to protein concentrations in milligrams per milliliter.

gible. Measurements from scanner recordings were carried out as described by Schachman and Edelstein (1966).

The partial specific volume, $\bar{v}$, of rabbit H, L, and Fd fragment was calculated from their published amino acid compositions (Crumpton and Wilkinson, 1963; Cebra *et al.*, 1968) and the partial specific volumes of the amino acids (Cohn and Edsall, 1943).

Protein concentrations were determined spectrophotometrically at 280 nm, with correction for light scattering. This correction was generally small. Extinction coefficients were determined by relating protein dry weight to absorbancy at 280 nm when sufficient material was available. Differential refractometry, using Rayleigh interference optics in the analytical ultracentrifuge, was used when only small quantities of protein were available (Babul and Stellwagen, 1969). The $\epsilon_{280}^{1\%}$ in dilute buffer was found to be 15.5 ± 0.1 for native anti-DNP (3 determinations by dry weight), 15.4 ± 0.2 for anti-DNP heavy chain, and 13.2 ± 0.1 for anti-DNP light chain (2 determinations in each case by refractometry). The latter value was used for both light-chain monomer and dimer since both have virtually identical $\epsilon_{280}^{1\%}$ values (Björk and Tanford, 1971b). $\epsilon_{280}^{1\%}$ values of normal rabbit IgG and its H and L chains used were 13.8, 14.4, and 12.1, respectively (Björk and Tanford, 1971a,b).

Results

Preparation and Purity of Anti-DNP Heavy Chain, Light Chains, and Fd Fragment. Rabbit anti-DNP H chain, prepared as described by Björk and Tanford (1971a), sedimented in the analytical ultracentrifuge as one major species with $s_{20,w}$ = 6.0 S at 3 mg/ml in 0.02 M sodium acetate (pH 5.5)–0.1 M NaCl (Figure 1a). Similar experiments at lower ionic strength (0.02 M sodium acetate, pH 5.5) gave similar results with the exception that the slight leading edge on the major peak was missing. Since aggregation appears to be minimal in this solvent, all binding measurements made with H chain were in 0.02 M sodium acetate (pH 5.5). Immunochemical analysis for the presence of L chain or intact IgG with goat anti-rabbit L chain by Öuchterlony immunodiffusion is seen in Figure 2a. A just barely visible line was seen with 0.1 mg/ml of IgG while a 2 mg/ml concentration of H chain gave no such line. By this criterion, the H chain preparation has less than 3–5% IgG contamination by weight. Thus, by sedimentation velocity and immunochemical analysis anti-DNP H chain was minimally contaminated with intact antibody or light chain and behaved in a manner similar to normal rabbit H chain (Björk and Tanford, 1971a).

Anti-DNP light chain renatured from 1 M propionic acid followed by chromatography on Sephadex G-75 in 0.02 M sodium phosphate–0.1 M NaCl (pH 7.0) separated into two major peaks (L_1 and L_2). Each peak was rechromatographed on a smaller column of G-75 in the same solvent and found to move as a single component. The results were essentially identical with those obtained with normal rabbit L chain (Björk and Tanford, 1971b; Figure 1).

Immunochemical analysis of unfractionated light chain for heavy chain contamination with goat anti-Fc serum is seen in Figure 2b. Less than 2% H chain contamination is seen with this antiserum. Similar results were also obtained with goat anti-rabbit Fd serum. Furthermore, when the L chain preparation was applied to the Sephadex G-75 column, only a small proportion (2–3%) of the total A_{280} units appeared in the void volume. Since both H chain and intact antibody eluted at or near the void volume, this result is in good agreement with the immunochemical results.

Fd fragment prepared by the procedure of Fleischman *et al.* (1963) showed one major component that sedimented with $s_{20,w}$ = 3.5 S (3.0 mg/ml) along with a minor trailing boundary which represents about 16% of the total protein (Figure 1b). Immunodiffusion against goat anti-L serum is seen in Figure 2c. Fd fragment appears to have no more than 12–15% Fab contamination by weight. Immunoelectrophoresis against goat anti-Fd supports the assertion that Fab and not free L chain was the contaminant.[3] Although the degree of impurity is somewhat higher than is desirable, it is still small enough to have little influence on the interpretation of binding measurements.

[3] These results along with a more detailed physical characterization of Fd fragment will be reported elsewhere (Huston, Björk, Painter, and Tanford, in preparation).

TABLE I: Molecular Weights of Isolated Anti-DNP Heavy and Light Chains at Near-Neutral pH as Determined by Sedimentation Equilibrium.[a]

Protein	Solvent	Molecular Weight
Heavy chain[b]	0.02 M acetate (pH 5.5) + 0.1 M NaCl	113,000
Light-chain monomer (L_1)	0.02 M sodium phosphate (pH 7.0) + 0.1 M NaCl	26,000[c]
Light-chain dimer (L_2)	0.02 M sodium phosphate (pH 7.0) + 0.1 M NaCl	39,700
Fd fragment[b]	0.02 M sodium acetate (pH 5.5) + 0.1 M NaCl	43,000

[a] Heavy-chain and light-chain monomer were meniscus depletion runs; light-chain dimer was a conventional low speed run. [b] Temperature was 5°; all others near 20°. [c] Slight upward curvature near the cell bottom.

Molecular Weights. Molecular weight measurements are summarized in Table I. Those for H and L chain were similar to those reported previously for normal rabbit IgG, *i.e.*, H chain was found to be a dimer and the two L chain fractions were found to represent populations of L chain in which the monomer–dimer equilibrium strongly favors monomer (L_1) and dimer (L_2), respectively. The Fd fragment was found to have an apparent molecular weight of 43,000–45,000. This value is somewhat lower than might be expected for an Fd dimer based on the known molecular weights of Fab (48,000, Noelken *et al.*, 1965) and L (22,000–23,000, Small and Lamm, 1966). The low value very likely reflects the presence of a smaller, possibly monomeric, component. The sedimentation velocity studies (Figure 1b) lend support to this conclusion. Since the protein concentrations used for hapten binding studies were greater than those used in the molecular weight measurements, we have assumed that Fd exists largely as a dimer in the hapten binding studies, using a molecular weight of 50,000 for the calculation of the molar protein concentration.

Hapten Binding Studies by Equilibrium Dialysis. The binding of [H^3]DNP-Lys to mildly reduced and alkylated anti-DNP was measured at 5° in 0.02 M sodium acetate, pH 5.5. These conditions were chosen so that average binding constants ($\bar{K}_0$) of intact antibody and its isolated chains could be compared under conditions where H chain dimer is most stable. Figure 3 shows a Scatchard plot for the binding of [H^3]DNP-lysine to mildly reduced and alkylated antibody. Results of two independent experiments are shown. Considering the numerous experimental difficulties involved in such measurement at these low protein and hapten concentrations, the agreement between these two separate experiments is good. The average values obtained for the two experiments are $\bar{K}_0 = 3.3\ (\pm 1.5) \times 10^8\ M^{-1}$ and $n = 1.9 \pm 0.1$, in good agreement with those reported for similar preparations of rabbit anti-DNP antibody (Eisen and Siskind, 1964; Jaton *et al.*, 1968).

Figure 4 shows binding data obtained for H-chain dimer and Fd dimer under conditions identical with those above. Because of the high protein concentrations used in these experiments it was not necessary to add 0.1% normal IgG to prevent protein absorption to the cell or dialysis membrane. The two preparations gave virtually identical results with $\bar{K}_0 = 6 \times 10^4\ M^{-1}$ and 1.0 ± 0.1 hapten binding sites/mole of protein. An experiment at a fivefold higher hapten concentration led to no further increase in r, confirming that all

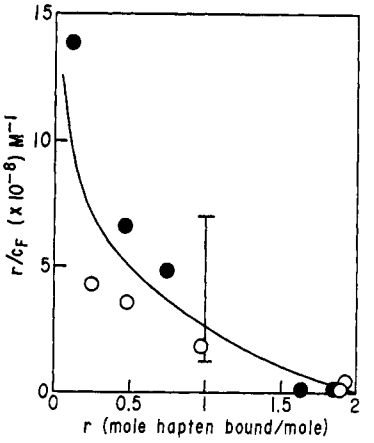

FIGURE 3: Scatchard plot of equilibrium dialysis data for mildly reduced and alkylated anti-DNP *vs.* [H^3]DNP-lysine at 5°, in 0.02 M sodium acetate (pH 5.5) containing 0.1% normal rabbit IgG. Two independent experiments are shown, at 1.0 and 2.0 × 10^{-7} M anti-DNP, respectively. It should be noted that the high value of the binding constant leads to accurate values for r, but to very large error in the evaluation of r/c_F, and that this error is greatly emphasized in this method of plotting. The resulting error in the determination of $\overline{\Delta G_u^\circ}$ is not very large: the error bar in the figure corresponds to the uncertainty of ±0.5 kcal/mole assigned to this quantity in Table III.

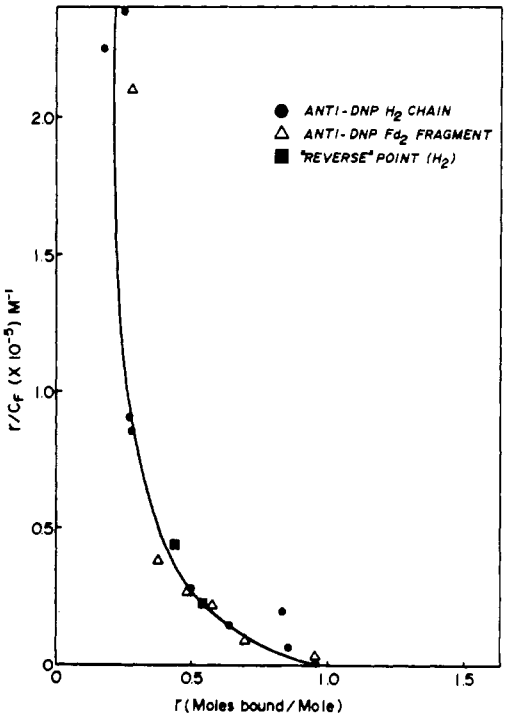

FIGURE 4: A Scatchard plot of the binding of [H^3]DNP-lysine to anti-DNP heavy chain and Fd fragment in 0.02 M sodium acetate (pH 5.5) at 5°. H chain (●—●) and Fd (△—△) concentrations, 1.14 × 10^{-5} M and 4.2 × 10^{-5} M, respectively. H chain "reverse" points (■) obtained by having protein and hapten initially on the same side of the dialysis membrane.

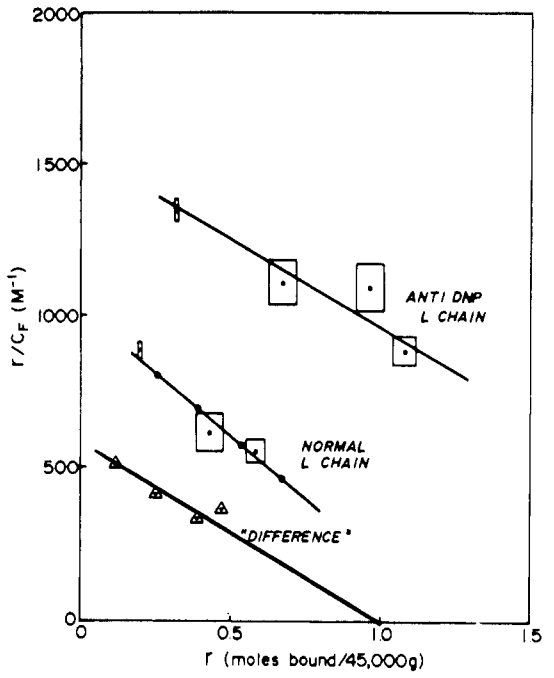

FIGURE 5: A Scatchard plot of [^{3}H]DNP-lysine binding by unfractionated rabbit light chains (L) in 0.02 M sodium acetate (pH 5.5)–0.1 M NaCl at 5°; anti-DNP L and normal L concentrations 36.4 mg/ml and 37.4 mg/ml, respectively. The data points represent the average of two replicate experiments while the open squares are the average range of error. The "difference" curve (Δ—Δ) is anti-DNP L data corrected for nonspecific binding of normal L.

available binding sites had been saturated. At free hapten concentrations above 0.8×10^{-4} M, small corrections ($r \simeq 0$–0.3) were made for nonspecific binding of hapten to normal H chain and Fd dimer.

Binding experiments with L chain proved more difficult because of its intrinsically low affinity for hapten. In order to achieve easily measured differences (> 10%) between nonprotein and protein sides of the dialysis membrane, L-chain concentrations of about 10^{-3} M were employed. Figure 5 shows a Scatchard plot of binding data for unfractionated (*i.e.*, a mixture of L_1 and L_2) anti-DNP L chains and a similar preparation of normal L chains. Specific L chain clearly bound 0.45 mole more hapten than did nonspecific light chain at a free hapten concentration of about 10^{-3} M. The observed difference cannot be the result of binding by contaminants, such as intact antibody or heavy chain. The level of contamination was not only too low to account for the observed difference but the affinity of the L-chain preparation for DNP-lysine was 2–3 orders of magnitude lower than that expected for heavy chain or reconstituted antibody (Painter *et al.*, 1971). Similar binding experiments performed with L monomer and L dimer are summarized in Table II. Although both forms of L chain bind significantly more hapten than their normal L chain counterparts, the monomeric form appears to bind hapten slightly better than the dimer. This difference is probably simply a consequence of L chain heterogeneity, with L_2 representing a fraction of light chains arising from a lower affinity antibody population. An alternative possibility could be that the process of dimer formation interferes to some extent with hapten binding, but this is unlikely because of the high L chain concentrations required to obtain binding data of sufficient precision. Under such conditions L_1 has been shown to dimerize (Björk and Tanford, 1971b), so that both L_1 and L_2 are likely to be dimeric in the binding studies.

TABLE II: Binding of [^{3}H]DNP-lysine to Rabbit Light (L) Chain at 5°.[a]

L-Chain Species	Final Protein Concn (mg/ml)	Free Hapten Concn (mM)	r (moles bound)/ 45,000 g[b]
L_1 (anti-DNP)	27.0	1.62	1.39 ± 0.06[c]
L_1 (normal IgG)	29.1	1.81	0.94 ± 0.04
L_2 (anti-DNP)	27.5	1.75	1.22 ± 0.05
L_2 (normal IgG)	22.1	1.76	1.00 ± 0.04

[a] Determined by equilibrium dialysis in 0.02 M sodium acetate (pH 5.5)–0.1 M NaCl. [b] Average of 2 determinations. [c] Standard deviation.

The limited solubility of [^{3}H]DNP-lysine at pH 5.5 made complete saturation of L-chain sites impossible. As a result the stoichiometry of the reaction is uncertain and the data represented by the triangles in Figure 5 can be extrapolated to yield 1 or 2 sites/45,000 g of light chain. The $\bar{K}_0$ for "specific" binding can be roughly estimated to be 500 M^{-1} from the calculated difference in binding between normal and antibody L chain if a value of 1 site/dimer is assumed. This value represents a maximum possible value of $\bar{K}_0$. If one assumes 2 sites/dimer then a lower value of approximately 200 M^{-1} is obtained. For these reasons, a range of 200–500 M^{-1} is recorded in Table III as a reasonable estimate of the anti-DNP L-chain binding constant for specific hapten binding. The importance of these results is (1) that specific L-chain binding of hapten does occur and (2) that it contributes significiantly to the total unitary free energy of hapten binding (see Discussion).

Table III summarizes the results of [^{3}H]DNP-lysine binding to antibody and its chains. Utsumi and Karush (1964) have pointed out that the contribution of each chain to antibody binding is more obvious if one compares unitary free energies ($\Delta\bar{G}_u°$) rather than equilibrium constants. Table III shows that the calculated sum of $\Delta\bar{G}_u°$'s for each chain accounts (within experimental error) for all the free energy of binding found in the intact antibody. The relative contribution of H and L chain to hapten binding energy is about 60 and 40%, respectively, even though $\bar{K}_0$ for each differ by more than a factor of 100.

Difference Spectra of Bound and Free DNP-L-lysine. Figure 6 shows difference spectra obtained for protein-bound hapten and free hapten at 5°. The data have been expressed in terms of molar differences in extinction coefficients ($\Delta\epsilon_m$) in order to facilitate a comparison of intact antibody and its chains. The expected concentration of bound hapten under these conditions was calculated from the equilibrium dialysis data presented above. The results obtained for intact antibody are in good agreement with results obtained by Little and Eisen (1967b) for high affinity anti-DNP antibodies. The difference spectrum showed a positive change in molar absorbancy of 900–1000 at 470 nm with a minimum at 420 nm. A hypochromic *red* shift in the hapten spectrum of approximately 10 nm was observed. Little and Eisen have observed this to be a specific feature of anti-DNP antibodies.

TABLE III: Binding of [^{3}H]DNP-lysine to Rabbit Anti-DNP Antibody and Its Chains at 5°.[a]

Protein	$\bar{K}_0$ (M^{-1})	n (mole/mole)	$-\Delta\bar{G}_u^0$ (kcal/mole)
Mildly reduced and carboxymethylated anti-DNP	3×10^8	1.9 ± 0.1	13.0 ± 0.5[c]
Heavy-chain dimer	6×10^4	1.0	8.3 ± 0.3
Fd dimer	6×10^4	1.0	8.3 ± 0.3
L-chain dimer	200–500	$\geqslant 1$[b]	5.4 ± 0.6[d]
$H_2 + L_2$			13.7 ± 0.7[e]

[a] By equilibrium dialysis. [b] See Figure 5 and text for details. [c] See Figure 3. [d] The uncertainty in $\Delta\bar{G}_u^0$ is calculated on the basis of a possible $\bar{K}_0$ value of 100 M^{-1}, which we consider to be outside the limits of experimental error (*cf.* Figure 5). [e] The calculated sum of the unitary free energies for H- and L-chain dimers. It is likely that combination with H chain or Fd dimer can take place in two orientations, depending on which chain is used as combining site. If correction for this statistical factor is made, the intrinsic value of $-\Delta\bar{G}_u^0$ for H chain or Fd would become 7.9 kcal/mole, and the sum for H and L chain would become 13.3 kcal/mole. A similar correction would apply to the L chain if the maximal value for $\bar{K}_0$ (corresponding to $n = 1$) is used.

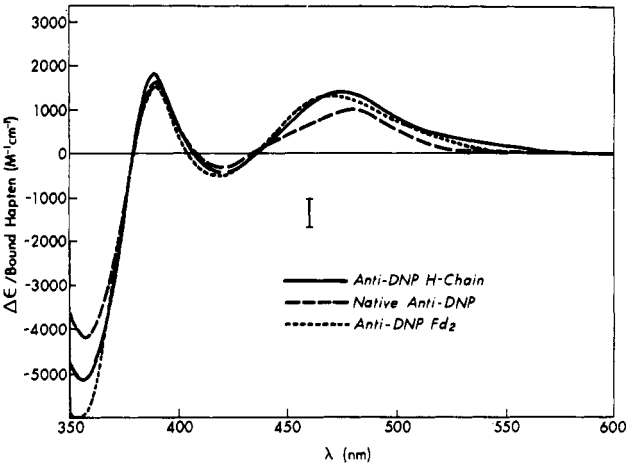

FIGURE 6: Difference spectra of protein-bound and free DNP-lysine in 0.02 M sodium acetate (pH 5.5) at 5° for native anti-DNP (- - -) at 2×10^{-5} M; anti-DNP H_2 (—) at 1.2×10^{-5} M; and anti-DNP Fd fragment (· · ·) at 1.42×10^{-5} M. Total DNP-lysine concentration 1.8×10^{-5} M except H_2 which is 8×10^{-5} M. Vertical bar represents the limits of uncertainty of $\Delta\epsilon_m$.

As Figure 6 shows, anti-DNP H chain as well as Fd fragment gave results which are virtually identical to the intact antibody. Normal H chain had only a small effect on the hapten sepctrum. In contrast to the red shift for specific H chains a slight hypochromic *blue* shift was observed which is characteristic of DNP-lysine binding to such "nonspecific" proteins as serum albumin (Carsten and Eisen, 1953).

Similar measurements for L chain could not be carried out because the high hapten concentrations required to generate a sufficient proportion of bound hapten gave rise to too high a total absorbancy to permit measurements to be made. Since H-chain binding accounts quantitatively for the difference spectrum observed with intact antibody, binding to L chain would presumably not make an additional contribution.

The molar absorption spectra of bound DNP-lysine confirm the difference spectral results. This method, unlike the difference spectral method, allows direct measurement of the bound-hapten concentration and absorbancy and is not subject to errors involved in the addition of precise aliquots of concentrated hapten to each cuvet. Since the free hapten concentrations in each cuvet are the same by virtue of the dialysis step, the difference in absorption between the two sides of membrane yields the absorption of protein-bound hapten. The molar absorption spectra of DNP-lysine bound to antibody H chain and Fd fragment gave results which are in reasonable quantitative agreement with the spectra obtained for hapten bound to native antibody. The observed molar extinctions at λ_{max} (373 ± 2 nm) are 14,200 ± 300, 14,800 ± 400, and 15,200 ± 400 for native antibody, anti-DNP Fd fragment, and anti-DNP H dimer, respectively. When compared with free DNP-lysine, (17,530 at λ_{max} 363) these results confirm the hypochromic red shift observed in the difference spectra. The value obtained with native anti-DNP is also in good agreement with previously reported values (Eisen and Siskind, 1964).

Both the difference spectra and the bound-hapten absorption spectra were obtained under conditions where more than 50% of binding sites were filled. The observed spectral shifts caused by specific H dimer and Fd dimer thus cannot be the result of contamination by intact antibody (or Fab) since the degree of contamination is not large enough.

On the basis of indirect evidence Little and Eisen (1967b) have attributed the hapten red shift observed in the binding of DNP-lysine with anti-DNP to an interaction of the hapten with a tryptophan residue(s) at the antibody site. Our observation of a similar shift in the binding of DNP-lysine to anti-DNP H chains suggests that the H chain predominantly donates the tryptophan residue(s) to the binding site of the intact antibody. A limited role by L chain in this regard cannot, of course, be completely ruled out.

ORD Spectra. The ORD spectra (220–260 nm) for intact antibody and anti-DNP H chain dimer, shown in Figure 7a, agree closely with similar data for normal rabbit IgG and H chain dimer (Björk and Tanford, 1971a). The spectrum for the intact antibody is also in good agreement with that reported by Steiner and Lowey (1966) for a preparation of similar affinity.

On the other hand, the L-chain ORD spectra, shown in Figure 7b, differ somewhat from those for normal rabbit L chains. Both L_1 and L_2 gave a somewhat smaller mean residue rotation than their normal rabbit counterparts. The difference is most apparent for L_2, which gave $[m'] = -1450$ at the 225-nm trough, as compared to -1840 for normal rabbit L_2 (Björk and Tanford, 1971b). These variations from normal IgG chains are not too surprising, when one considers that anti-DNP probably represents a more restricted population of immunoglobulins than pooled nonspecific immunoglobulin. In this regard, it should be noted that intact anti-DNP and normal IgG exhibit similar quantitative differences in their ORD spectra while the general features of the two spectra are indistinguishable (Steiner and Lowey, 1966; Painter *et al.*, 1972).

Another new observation, seen in Figure 7, is that the ORD spectrum of L_1 shows a distinct dependence on protein

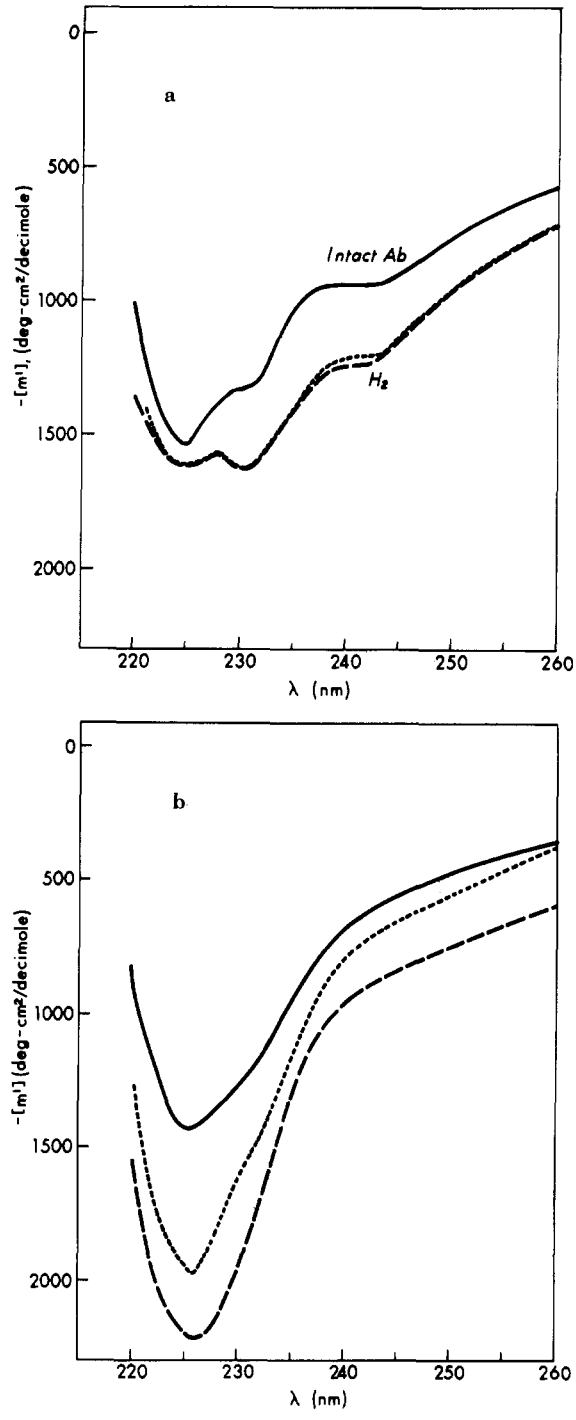

FIGURE 7: ORD spectra of anti-DNP and its chains. (a) Spectrum of mildly reduced and carboxymethylated anti-DNP (or native anti-DNP) in 0.02 M NaCl pH 5.5 (—); 25–26°; H-chain dimer 1.78 mg/ml (- - -) in 0.02 M sodium acetate (pH 5.5) at 8°; H-chain dimer plus 4×10^{-4} M DNP-aminocaproate (· · ·) under identical conditions. (b) Light-chain dimer (L_2) at concentrations ranging from 0.2 to 22 mg/ml and light-chain monomer (L_1) at concentrations of 0.2 mg/ml (- - -) and 22 mg/ml (· · ·). Solvent: 0.02 M NaCl (pH 5.5); 25–26°.

concentration with the mean residue rotation becoming less negative with increasing concentration. L_2, on the other hand, showed no such dependence over a 100-fold range of concentration. The fact that the ORD spectrum of the monomeric form approaches that of the dimer as its concentration is raised suggests that the difference between the L_1 and L_2 spectra is, in part, a result of the dimerization process rather than an intrinsic property of a subclass of light chains.

It is well established that the weighted sum of the $[m']$ for an equimolar ratio of H and L chains (*i.e.*, $[m']_{H\text{-}L} = 0.32 \cdot [m']_L + 0.68[m']_H$) does not account quantitatively for the ORD spectrum observed for native immunoglobulin (Dorrington *et al.*, 1967; Stevenson and Dorrington, 1970; Björk and Tanford, 1971c). This has been confirmed here as well. These observations have been interpreted to mean that a conformational change has occurred when the chains are separated. It has been proposed that such a change might account for the lowered affinity of H chain (Utsumi and Karush, 1964; Genco *et al.*, 1968). If a conformational change does indeed occur in the binding site regions of the isolated chains and is the cause of their low affinities, then the energy gained in binding of hapten might induce a change in ORD toward a more native state. ORD measurements were therefore performed on specific heavy chains in the presence of excess hapten.

For these studies DNP-aminocaproate was used in place of DNP-lysine because (1) it has no intrinsic optical activity, and (2) it binds to anti-DNP as well as, or better than, DNP-lysine (Eisen and Siskind, 1964). Figure 7a shows that the ORD spectrum of specific heavy-chain dimer was not affected by the presence of 4×10^{-4} M DNP-aminocaproate. Essentially all H-chain sites were saturated under the conditions used in this experiment. Similar experiments (not shown) with antibody L monomer and L dimer also gave no evidence of ORD changes in the presence of DNP-aminocaproate even when concentrations as high as 0.9×10^{-3} M hapten were employed. By this criterion there is no evidence for a conformational change when hapten binds to the isolated antibody chains. This result is consistent with the finding that the $\Delta\bar{G}_u^\circ$ of hapten binding to the isolated chains of anti-DNP antibody are additive and account for all the binding energy of the original antibody. It indicates that the conformations of the binding site regions are not substantially different in the free chains and in intact antibody, and suggests that the experimentally observed differences arise from portions of the H and L chains that do not contribute to the binding site.

Discussion

Physical Properties and Number of Binding Sites. The molecular weights and other physical properties of rabbit anti-DNP heavy and light chains have been found to be similar to those previously reported for the polypeptide chains from normal rabbit IgG (Björk and Tanford, 1971a,b). In particular, the dimeric state of Fd has been confirmed, indicating that the heavy-chain dimer is held together by noncovalent forces in both the Fc and Fd regions of the molecule. On the basis of this finding Björk and Tanford (1971a) suggested that one of the Fd regions in the H chain or Fd dimer might be structurally equivalent to the L chain of the intact antibody, and they therefore predicted that only one hapten binding site might be accessible in H_2 or $(Fd)_2$. The results reported in this paper indicate that this is indeed true. A similar prediction was made by these authors for the light-chain dimer. Unfortunately the technical difficulties involved in the light-chain binding measurements allow no definitive statement to be made, but the data are consistent with 1 site per L dimer. Previous quantitative binding measurements have generally found a fractional number of sites (about 0.5/dimer) for H chain with a higher $\bar{K}_0$ than that found here (Weir and Porter, 1966; Haber and Richards, 1966; Jaton *et al.*, 1968). One exception is Utsumi and Karush (1964) who have reported

data which are consistent with 1 site per dimer. The fractional number of sites observed previously was probably due to experimental difficulties, such as partial denaturation and aggregation of the preparations. Alternatively, a portion of low affinity sites may have been missed if hapten concentrations were not sufficient to saturate all the available sites. The latter possibility has been ruled out in the present case since a fivefold increase in hapten concentration led to no measurable increase in the observed number of sites. It is conceivable, of course, that our preparation may also have been partially denatured, and our result of 1 site/dimer may be purely accidental. Fluorescence quenching measurements, however, suggest that this is not the case.[3] The maximal quenching observed shows that close to 100% of the H chains in our preparations were affected by the interaction with antigen.

The ORD spectra of anti-DNP H and L chains show the same general features as those seen previously for the normal chains. In spite of the overall similarities observed there do exist small but significant differences in absolute rotation. Steiner and Lowey (1966) have shown an apparent correlation between antibody specificity and ORD, and have indicated that allotypic variations in the immunoglobulins or net charge are not involved. This implies that the variable amino-terminal regions of the H and L chains are responsible for this phenomenon, and this is consistent with our observations. There was a greater difference between the [m'] values of anti-DNP L_1 and L_2 chains and normal L_1 and L_2 chains (10–25%) than between those of anti-DNP H chains and normal H chains, which were barely outside experimental error (~10%). This may be taken as a reflection of the fact that a greater portion of the L chain (50%) contains a variable amino acid sequence than does the H chain (25%).

The calculated ORD spectrum for an equimolar mixture of free light and heavy chains generally does not regenerate the native IgG spectrum, implying that a conformational change of some kind has occurred within the H and/or L chains when they are separated (Steiner and Lowey, 1966; Dorrington *et al.*, 1967; Björk and Tanford, 1971c; Stevenson and Dorrington, 1970). The results presented here for anti-DNP antibodies are in agreement with these previously observations. In spite of the ORD evidence which suggest conformational changes in the isolated chains, we have shown that the unitary free energy of the chains accounts for the binding of hapten by the intact antibody. Since specific hapten binding is likely to be very sensitive to small conformational changes in tertiary structure near the active sites, the ORD and binding results can be reconciled if one postulates that the conformational changes reflected in the ORD spectra are not associated with the active site regions. Further support of this concept is provided by the finding that the presence of bound hapten had no effect on the ORD spectra of the isolated chains. Thus, the physical binding of hapten to heavy or light chain does not force their conformations (as judged by ORD) into a more native one. The binding measurements, in fact, rule out such an eventuality since such a process would require considerable energy and thus require the free energies of the chains to be nonadditive Thus it can be concluded that the changes in tertiary structure reflected in the ORD data occur in regions isolated from the antigen combining site.

Free Energy of Hapten Binding. The average hapten binding constant ($\bar{K}_0$) observed for heavy-chain dimer is about 5000-fold lower than that observed for native anti-DNP. The binding constant observed for heavy chain is also considerably lower (by a factor of about 50) than those observed previously for anti-DNP H preparations of similar affinity (Weir and Porter, 1966; Haber and Richards, 1966; Jaton *et al.*, 1968). The source of this discrepancy is very likely a result of the low number of binding sites (0.5/dimer) found by previous workers and used to calculate $\bar{K}_0$. Snce $\bar{K}_0$ is defined as $1/c_f$ at 50% saturation such an error would lead to a value of $\bar{K}_0$ which is higher than the true value. The error is further accentuated by the marked curvature in the Scatchard plot caused by heterogeneity of the binding sites. Thus, in the present case, if data had not been collected at sufficiently high hapten concentrations in order to insure complete saturation, it could have been concluded that H_2 had 0.5–0.6 site/mole with an apparent $\bar{K}_0$ of about 5×10^5 M^{-1}. Since the presence of denatured protein should lower the apparent number of sites but not $\bar{K}_0$ (assuming, of course, that selective denaturation of heavy chain with respect to affinity does not occur), the high values of $\bar{K}_0$ obtained in the past suggest that these workers did not use hapten concentrations which were sufficient to saturate all available sites.

The L-chain binding results strongly suggest that anti-DNP L chain binds hapten specifically but with very low affinity. This confirms previous reports of antigen binding by specific light chain (Yoo *et al.*, 1967; Goodman and Donch, 1965; Mangalo and Raynaud, 1967). The direct participation of L chain at the antibody active site indicated by these results is also in agreement with numerous affinity labeling experiments which show labeling of both chains (Wofsy *et al.*, 1967; Good *et al.*, 1968; Thorpe and Singer, 1969). The L-chain binding data reported here are the first which are quantitative enough to permit an estimation of $\bar{K}_0$ from 200 to 500 M^{-1}. This is the first report to our knowledge in which quantitative $\bar{K}_0$'s for H and L chain from the same antibody preparation have been measured.

The most important result of this work is the finding that the $\Delta\bar{G}_u^\circ$'s of the H and L chains are additive and account for the $\Delta\bar{G}_u^\circ$ of hapten binding by the native antibody. This result has several important consequences. (1) Both chains donate direct contact regions to the antibody active site. (2) The conformations of the binding site regions of the free chains are essentially unaltered after the chains have been separated and then renatured at pH 5.5. These consequences argue against the hypothesis that the L chain is not directly a part of the binding but serves to orient the H chain so that it can bind more avidly in the native state (Genco *et al.*, 1968).

The direct binding measurements made here confirm the results of more indirect methods such as affinity labeling (Good *et al.*, 1968; Wofsy *et al.*, 1967; Thorpe and Singer, 1969), as well as numerous recombination studies of heavy and light chains (Fraňek and Nezlin, 1963; Olins and Edelman, 1964; Roholt *et al.*, 1964; Painter *et al.*, 1972). It should be noted however that, because of antibody heterogeneity, such approaches cannot unequivocally determine if both chains are involved in a single antibody molecule. It is conceivable that in a heterogeneous population some molecules may have an active site donated entirely by a heavy chain while others are donated entirely by the light chain. No such ambiguity pertains to the results reported in this paper, and they indicate that, for the majority of antibody molecules, both chains are involved. The evidence for this follows. (1) At least 85–90% of the H chain population binds DNP-L-lysine specifically. The fraction of active L chains could not be assessed because saturation of the binding sites could not be achieved, but it is certainly no less than about 50%. (2) If L-chain dominant antibodies were significant, L chain with

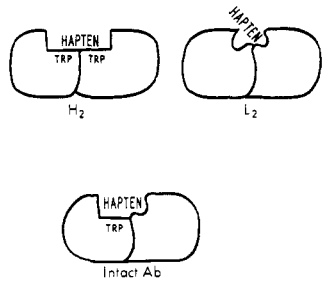

FIGURE 8: A schematic representation of the relative contributions made by heavy (H_2) and light chains (L_2) to the anti-DNP (Ab) binding site. The regions of the chains depicted represent those in the immediate vicinity of the active site. The presence of two tryptophans (Trp) at the H_2 site does not necessarily imply they *both* interact with hapten.

binding affinity comparable to H chains would have been expected, and the sum of the observed unitary free energies of binding would have exceeded the unitary free energy for binding to native antibody. (3) As will be shown in the following paper (Painter *et al.*, 1972), hybrid recombinants of anti-DNP H chain with normal nonspecific L chains show that most H chains have a binding site for DNP-lysine when their mutual interaction in H chain dimer no longer exists.

It may also be noted that affinity labeling studies with homogeneous mouse myeloma proteins possessing anti-DNP activity show labeling of both chains. This suggests that both chains are in relatively close contact with the active site region on the native protein (Haimovich *et al.*, 1970).

The most plausible picture at our present level of knowledge is one in which there exists a population of molecules, a small proportion of which might be H or L dominant. The majority, however, would be expected to have both chains participating at the antibody combining site.

Hapten Difference Spectra Association with Specific Binding. The implication of tryptophan at the combining site of anti-DNP H chain (and Fd) which is suggested by the hapten difference spectra is of considerable interest. Such spectral changes are likely to be the result of short-range electronic interactions and thus are critically dependent on orientation factors as well as environmental factors. The remarkable similarity between the spectral perturbations caused by hapten binding to H_2 (or Fd) and intact antibody suggests (1) that a majority of the tryptophan responsible for such perturbation by the native antibody is located in the Fd region of the H chain and (2) that the H-chain, active-site region is probably similar in its environment to that found in the native antibody. These direct observations are also consistent with the finding that high-affinity anti-DNP antibodies have on the average 1.5 more tryptophan residues than low-affinity anti-DNP (McGuigan and Eisen, 1968), and with the higher extinction coefficients at 280 nm for anti-DNP antibodies (as compared with nonspecific IgG) found here and reported previously (Steiner and Lowey, 1966). Furthermore, McGuigan and Eisen have found the increased tryptophan content of high affinity anti-DNP to be entirely associated with the Fd region while the tryptophan contents of the L chains in high and low affinity anti-DNP were not significantly different. Tryptophan has also been implicated in the combining site of antibodies to fluorescein (Werner and Cathou, 1971) raising the possibility that site-associated tryptophan might be a general feature of antibodies to aromatic compounds.

Conclusions

Figure 8 presents a schematic model of antibody and its free chains which is consistent with all the results presented. The following characteristics of such a model follow. (1) The heavy chain dimerizes in the Fd region in such a manner as to form a single hapten accessible site. (2) Although two sites/L dimer has not been ruled out as a possibility, the L chain, like H_2, could have 1 site/dimer. (3) In a single antibody molecule, *both* H and L chains contribute contact residues to the hapten binding site and in the case of H_2, tryptophan has been identified as one such possible amino acid residue. (4) The regions of both chains involved in making up the active site of the antibody undergo no change in conformation which is sufficient to alter hapten binding energies. The ORD spectra of the isolated chains suggest that changes do occur as a result of separation but these must be in regions which are essentially independent of the active site.

It has been suggested previously that a multitude of different antibody specificities could be generated from a relatively small number of H and L chains through random combination of H and L chains with different specificities for portions of antigenic determinants (Edelman and Gally, 1964). The results of this paper, indicating that the free energy of binding of the intact antibody is the sum of contributions from the H and L chains, are consistent with this possibility.

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