Contributions of Heavy and Light Chains of Rabbit Immunoglobulin G to Antibody Activity. II. Binding Activities of Reconstituted Immunoglobulins†

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ABSTRACT: Heavy (H) and light (L) chains derived from rabbit anti-2,4-dinitrophenyl (anti-DNP) antibody and normal rabbit immunoglobulin G (IgG) were recombined in 1 M propionic acid or at pH 5.5, and reconstituted immunoglobulins isolated by gel chromatography. The reconstituted immunoglobulins, in all cases, had the physical–chemical properties expected for the structurally intact immunoglobulin. Hapten binding measurements indicated that IgG reconstituted from anti-DNP and nonspecific chains (hybrid molecules) bound hapten with an affinity similar to that of the anti-DNP chain used in the recombination, indicating that the nonspecific chains had no appreciable effect on the binding site regions of the specific chains. Recombination of specific L chain with specific H chain to form a homogeneous recombinant led to a 5- to 10-fold higher average hapten binding constant than a hybrid molecule containing specific heavy chain and nonspecific light chain. However the homogeneous recombinant bound hapten with an affinity which was still 2000-fold lower than that of intact antibody. Full affinity was recovered only if the interchain disulfide bonds connecting the H and L chains were not reduced prior to the denaturation and renaturation process. An exact matching of the original H and L chain partners is apparently required for the formation of a composite site capable of combining with the entire reactive surface of the hapten, but not for the formation of a structurally competent immunoglobulin. These results confirm previous results with free H and L chains which show that both chains play a direct role in binding antigen.

Antigen binding studies of reconstituted antibodies have generally shown that, while the gross IgG[1] structure is regained, antigenic binding affinity is substantially lowered (Edelman *et al.*, 1963; Fraňek and Nezlin, 1963; Roholt *et al.*, 1964; Hong and Nisonoff, 1966; Haber and Richards, 1966). Since heavy and light chains derived from the same antibody preparations yield a higher relative recovery of binding affinity than do recombinants of chains from antibodies of differing antigenic specificity (Roholt *et al.*, 1965a,b, 1966), it has been suggested that both heavy and light chains are involved in determining antibody specificity. An extension of this concept is the idea that the exact pairing of the heavy and light chains found in the parent molecules is required for full expression of binding activity.

Others, however, have suggested that the loss of binding activity by heterologous[2] recombinants is due to the failure of these recombinants to regain a truly intact immunoglobulin conformation (Stevenson and Dorrington, 1970; Grey and Mannik, 1965; Mannik, 1967). The recent work of Björk and Tanford (1971) argues against this possibility, having shown that all structural features of normal rabbit IgG appear to be completely regained after chain separation and recombination. The preceding paper (Painter *et al.*, 1972) is also inconsistent with this suggestion, since it shows that the free energy of association between an antibody and specific hapten can be completely accounted for in terms of the free energies of association between antigen and separated H and L chains derived from the antibody.

The purpose of this paper is to examine the structure of recombined molecules formed from heavy and light chains derived from rabbit anti-DNP and from normal nonspecific rabbit IgG, to reinvestigate their binding properties, and to compare them with the binding properties of the separated chains.

Materials and Methods

Methods for preparing rabbit anti-DNP antibody and for isolation of its heavy and light chains have been described in the preceding paper (Painter *et al.*, 1972).

Recombination of heavy and light chains, after their separation in 1 M propionic acid, was carried out by one of two methods. In the first, hereafter referred to as method I, the isolated chains were renatured separately by dialysis against 0.01 M sodium acetate buffer (pH 5.5). The heavy and light chains were then mixed (in a 1:1 molar ratio) in the cold and allowed to recombine for 20 hr. The total protein concentration was 0.8 mg/ml in a volume of 32 ml of 0.01 M sodium acetate buffer (pH 5.5). A second procedure, which is essentially that of Metzger and Mannik (1964), was used in some instances. In this method, hereafter referred to as method II,

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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).

[2] A homologous recombination is one in which the H and L chains are derived from the same antibody preparation whereas a heterologous recombination is one in which the H and L chains come from different immunoglobulin preparations. An autologous recombination is reserved for the case where the chains of a homogeneous immunoglobulin, such as a myeloma protein, are recombined.

heavy and light chains were mixed (1:1 molar ratio) while still in 1 M propionic acid and then dialyzed against 0.1 M sodium acetate (pH 5.5) for at least 30 hr with three changes of buffer.

After recombination had been allowed to proceed for at least 20 hr, the mixtures obtained by both procedures I and II were concentrated tenfold by ultrafiltration through a UM-10 membrane (Amicon Corporation, Lexington, Mass.), and dialyzed for 12 hr against 0.02 M sodium acetate (pH 5.5)–0.1 M NaCl. The recombinant mixtures were finally isolated by gel chromatography in the same solvent on a column (2.5 × 90 cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N. J.).

Sedimentation velocity measurements were performed at protein concentrations of 0.8–1 mg/ml in pH 5.5 0.02 M acetate–0.1 M NaCl buffer using a Beckman Model E analytical ultracentrifuge equipped with absorption optics and a uv photoelectric scanning system. All sedimentation velocity experiments were done at 60,000 rpm and 25° in a 2.5-deg, 12-mm double sector cell, with a monochrometer wavelength of 290 nm.

The percentages of heavy and light chain (by weight) in the final recombined products were determined by gel filtration of the completely reduced and carboxymethylated proteins (2 mg) on a column (1.5 × 85 cm) of Sepharose 6B in 6 M Gdn·HCl[3] containing 0.01 M sodium acetate (pH 4.75), essentially as described by Fish *et al.* (1969). The effluent was assayed spectrophotometrically at 280 nm. The extinction coefficients of the appropriate chains in 6 M Gdn·HCl were assumed to be identical with those in dilute buffer.

Extinction coefficients at 280 nm for recombined hybrids of heterologous heavy and light chains were determined by relating absorbancy at 280 nm to protein concentration. Protein concentrations were determined by a modified Nessler titration (Koch and McMeekin, 1924), assuming a nitrogen content of 16% by weight which is comparable with the value of 15.85 calculated from the amino acid composition of IgG (Crumpton and Wilkinson, 1963). Bovine serum albumin and native anti-DNP served as standard controls. The extinction coefficients at 280 nm for a 1% solution (weight/volume) in a 1-cm cell were 15.4 ± 0.6 and 14.4 ± 0.6 (average of 3 determinations) for the H^DL^N and H^NL^D recombinants, respectively. Values for native anti-DNP (15.5) and normal IgG (13.8) were used for the homologous recombinants.

Hapten binding measurements by equilibrium dialysis and fluorescence quenching, ORD measurements, and difference spectroscopy were performed as described in the preceding paper (Painter *et al.*, 1972).

Results

Efficiency of Recombination and Sedimentation Behavior. Figure 1 shows a typical G-200 elution pattern obtained with a 1:1 molar mixture of anti-DNP heavy and light chains. The chains in this case were recombined at pH 5.5 after being separately renatured (method I). The elution profile shows small amounts of aggregated material as a leading shoulder of the main recombinant peak along with a trailing free light chain peak. The percentage by weight of each component (as estimated by measuring the area under each peak and assuming a Gaussian distribution) was 15% aggregate, 75% recombined antibody (H^DL^D) and 10% light chain. The recombinant fraction was pooled as shown in Figure 1, and used for all subsequent measurements.

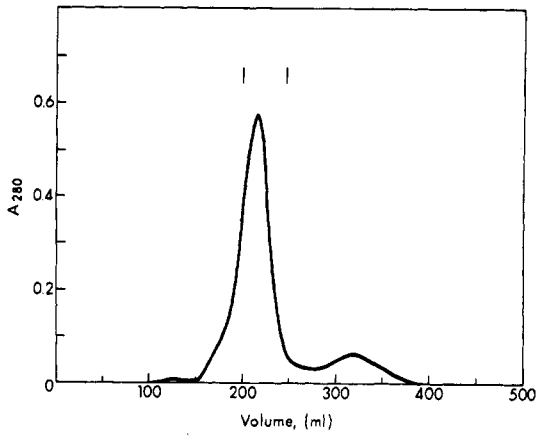

FIGURE 1: Gel chromatography on Sephadex G-200 of recombined anti-DNP H and L chains. Column dimensions 2.5 × 90 cm; solvent, 0.02 M sodium acetate (pH 5.5)–0.1 M NaCl; 25 mg of an equimolar mixture of H and L chains applied 30 hr after mixing at 8 mg/ml; vertical bars show fraction pooled for further study.

Sedimentation velocity studies performed with this fraction (1 mg/ml) with the photoelectric scanner, showed a single symmetrical boundary which sedimented at $s_{20,w}$ = 6.52 S. This value of $s_{20,w}$ is the same as that obtained for native mildly reduced and alkylated anti-DNP under the same conditions.

Results similar to those obtained with H^DL^D were found when heavy and light chains derived from different parent immunoglobulins were combined; *e.g.*, anti-DNP H and nonspecific L or *vice versa*. All results of this kind are summarized in Table I. This table also shows that the yield of recombinants and $s_{20,w}$ were the same when the chains were mixed in propionic acid and subsequently renatured (method II) as when method I was used. These results are in agreement with the observations of Björk and Tanford (1971) on recombined normal rabbit IgG. The similarity in per cent yields indicates that any H and L pair can form a stable immunoglobulin-like structure. The virtual identity of $s_{20,w}$ with that of the intact antibody, as well as the ORD results below, provide good evidence that a structurally intact four-chain immunoglobulin was obtained in all cases.

As a control for the hapten binding studies reported below, native anti-DNP, with its interchain disulfide bonds intact, was treated with 1 M propionic acid for 24 hr and then renatured and chromatographed on G-200 in a manner identical with the other recombination experiments. As seen in Table I, a lower yield (45–50%) of "recombined" product was obtained along with a corresponding increase in aggregated protein. (No elution peak at the position corresponding to light chains was, of course, observed.) The sedimentation coefficient of the nonaggregated material was identical with that of the native protein. The simplest explanation for the lower yields in this case is that polymer formation can occur when a heavy and light chain from two different IgG molecules form a stable intermolecular noncovalent bond and the chains of each molecule are still interconnected by disulfide bonds.

[3] Abbreviations used are: Gdn·HCl, guanidine hydrochloride; H^DL^D means a reconstituted immunoglobulin made up of anti-DNP heavy and anti-DNP light chains; H^DL^N refers to one made up of a pair of anti-DNP heavy chains and a pair of normal light chains; H^NL^D, normal heavy chain and anti-DNP light chain; H^NL^N, both chain pairs derived from normal rabbit IgG. DNP-lysine, ε-amino-DNP-L-lysine.

TABLE I: Yield and Sedimentation Properties of Immunoglobulin Recombinants Purified by Gel Chromatography.

Species	Method of Recombinations	$s_{20,w}$ (S)[b]	Yield of Recomb (%)[c]
Untreated, mildly reduced and alkylated anti-DNP		6.52	
Native anti-DNP[d] (S-S intact)	II	6.5	45–50
H^DL^D	II	6.50	75
H^DL^D	I	6.51	73
H^DL^N	I	6.45	81
H^NL^D	I	6.50	75
H^NL^N[e]	I or II	6.6	80

[a] In method I the chains are mixed at pH 5.5 while in method II the chains are mixed prior to removal of 1 M propionic acid by dialysis (see text for details). [b] $s_{20,w}$ at 0.8–1.0 mg/ml, pH 5.5. [c] Yield expressed as the weight percent of recombination as judged by Sephadex G-200 chromatography (see Figure 1). [d] S–S intact, refers to native antibody with all disulfide bonds, including interchain bonds intact. Others have their interchain S–S bonds reduced and carboxyamidomethylated. [e] Data of Björk and Tanford (1971).

Figure 2 shows an elution pattern for an H^DL^D recombinant obtained by chromatography of the completely reduced and carboxymethylated protein on a column of Sepharose 6B (1.5 × 85 cm) in 6 M Gdn·HCl–0.01 M sodium acetate (pH 4.75). Since many proteins, including IgG, exist as linear random coils in this solvent (Tanford, 1970), the quantitative separation of individual polypeptide chains according to their molecular weight can be accomplished and a rigorous value for unknown subunit weights can be determined. As Figure 2 shows, the recombined immunoglobulin gave two peaks in the dissociating solvent at positions expected for heavy and light

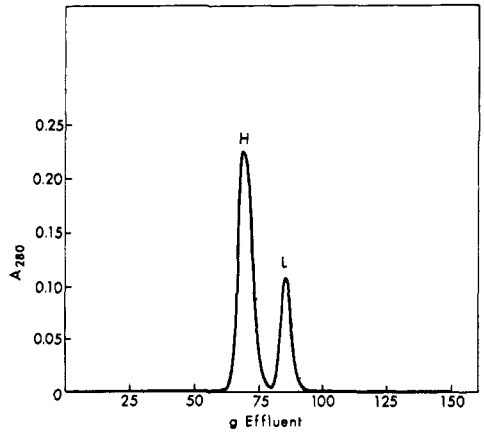

FIGURE 2: Gel chromatography of completely reduced and alkylated immunoglobulins on Sepharose 6B in 6 M Gdn·HCl–0.01 M sodium acetate (pH 4.75). Column dimensions, 1.5 × 85 cm; 2 mg applied in 0.1 ml; eluted at a rate of 2.50 g solvent/hr. The elution positions of the H and L chains were those expected for molecular weights of 50,000 and 23,000, respectively, based on a calibration curve obtained by chromatography of standards with known molecular weights.

TABLE II: The Heavy- and Light-Chain Compositions of Immunoglobulins Reconstituted at pH 5.5.[a]

Recombinant	Weight (%)[b]	
	Heavy Chain	Light Chain
H^DL^D	67	33
H^DL^N	68	32
H^NL^D	67	33

[a] Determined by gel chromatography on 6% Sepharose in 6 M Gdn·HCl after complete reduction and carboxymethylation (Figure 2). [b] The values for native IgG, based on molecular weights of 50,000 and 23,000 for heavy and light, are 68.5% heavy and 31.5% light.

chains. The proportions of each subunit (by weight) after correcting for the difference in extinction coefficients of heavy and light chains are 68% H and 32% L. These compare favorably with the values of 68.5% H and 31.5% L, based on the known molecular weights of rabbit heavy and light chains. Table II summarizes the results of similar experiments obtained for other reconstituted immunoglobulins. The heavy- and light-chain composition is in excellent agreement for both homologous and heterologous recombinants, providing additional evidence that the gross physical structure characteristic of native IgG has been regained in all cases. Similar results have been reported by a number of other workers (Edelman *et al.*, 1963; Olins and Edelman, 1964; Roholt *et al.*, 1964; Bridges and Little, 1971).

ORD Spectra. The preceding results show that a compact four-chain IgG molecule is reconstituted when heavy and light chains are recombined, but they are not sensitive to subtle changes in protein conformation. Optical rotary dispersion (ORD) provides a convenient probe for detecting such changes.

ORD spectra of homologous anti-DNP heavy- and light-chain recombinants are shown in Figure 3a along with the spectrum of intact anti-DNP. As seen, the agreement between the intact spectrum and that of H^DL^D, recombined by either method I or II, is excellent. The native anti-DNP control, which had been renatured with its interchain disulfide bonds intact, and chromatographed to remove aggregates, gave a mean residue rotation at 225 nm, which was slightly more negative than the spectra of the other recombinants or the untreated antibody. This difference is very likely due to the presence of a small quantity of aggregated protein, because there was a high percentage of aggregated protein found with this recombinant (see Table I) and gel chromatography might not have completely removed all traces of aggregate.

Analysis of the ORD spectra of the hybrid recombinants H^DL^N and H^NL^D (Figure 3b) is made somewhat more difficult by virtue of the nonidentity of the ORD spectra of native anti-DNP and nonspecific IgG (Steiner and Lowey, 1966; Painter *et al.*, 1972). Assuming that the formation of a conformationally competent immunoglobulin occurs by a random combination of any light and heavy chain, it is reasonable to expect a hybrid molecule to exhibit an ORD spectrum having the spectral features intermediate between those of native anti-DNP and nonspecific IgG. Furthermore, since the contribution of the heavy chain to $[m']$ is about double that of light chain (because heavy chain represents 68% of the total

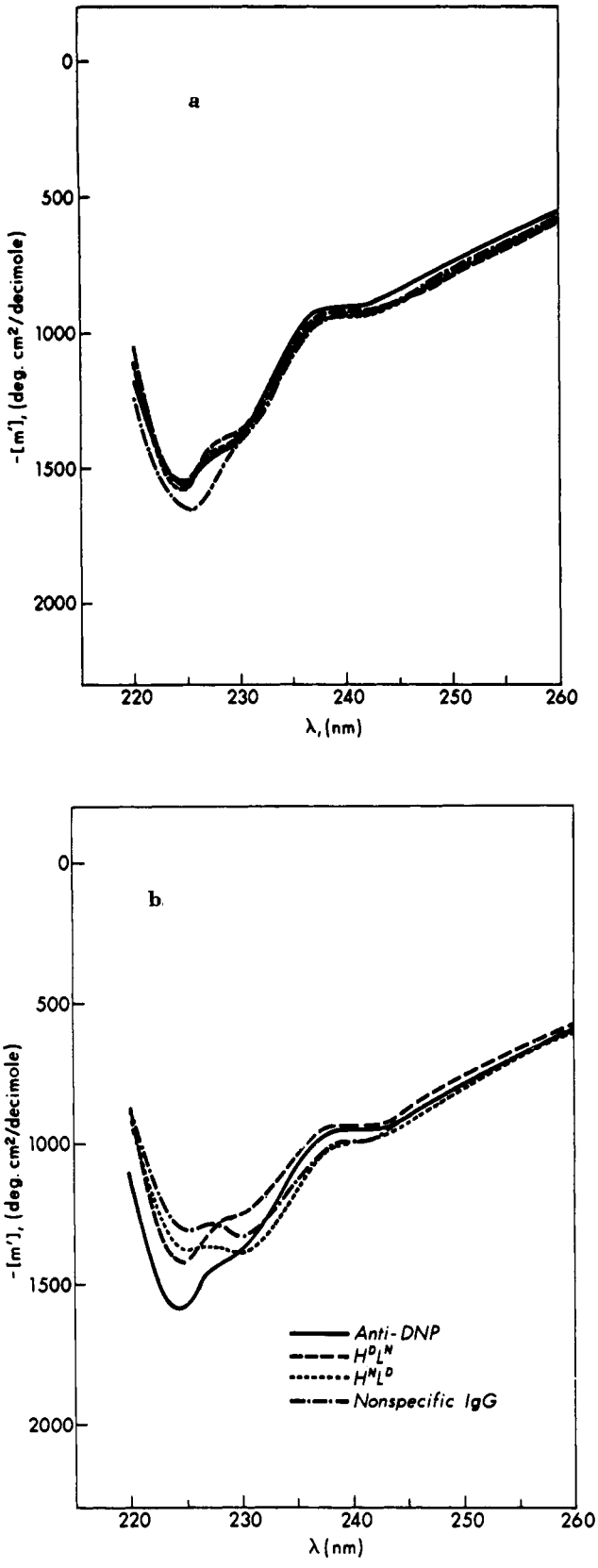

FIGURE 3: Optical rotatory dispersion (ORD) spectra of reconstituted immunoglobulins in 0.01 M NaCl (pH 5.5). Protein concentration, 0.15–0.20 mg/ml, 25–26°. (a) Homologous anti-DNP recombinants H^DL^D (recombined by method I)- - -, H^DL^D (recombined by method II)···, native anti-DNP renatured from 1 M propionic acid with intact disulfide bonds —·—, and mildly reduced and alkylated (or native) anti-DNP—. (b) ORD spectra of heterologous recombinants of anti-DNP and nonspecific rabbit chains, H^DL^N - - - and H^NL^D···. Mildly reduced and alkylated anti-DNP (—) and nonspecific IgG (—·—) spectra are shown for comparison.

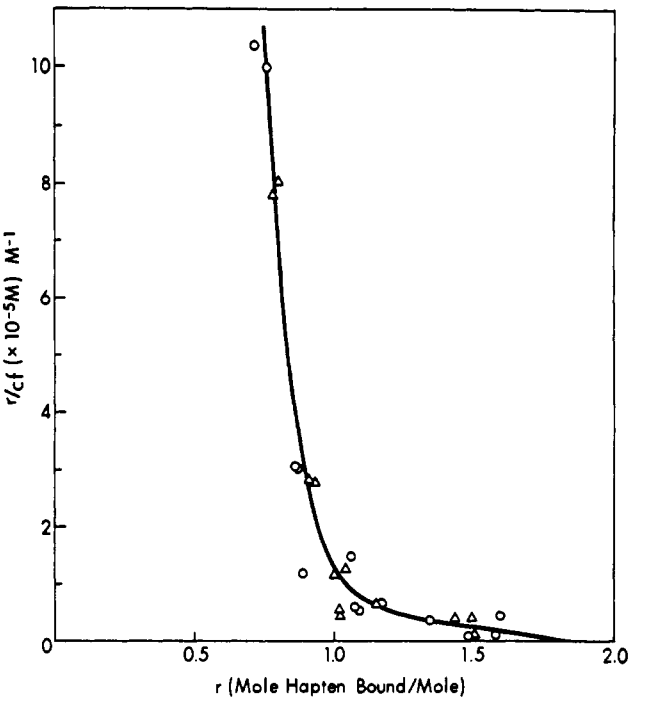

FIGURE 4: Scatchard plot of data obtained for the binding of [^{3}H]-DNP-lysine to homologous anti-DNP recombinants by equilibrium dialysis at 5° in 0.02 M sodium acetate–0.1 M NaCl (pH 5.5). H^DL^D (recombined by method I) O and H^DL^D (recombined by method II) Δ at protein concentrations that ranged from 0.35×10^{-5} to 3.2×10^{-5} M. Data corrected for small binding of hapten by nonspecific IgG.

IgG mass) the hybrid spectrum should more closely resemble the spectrum of the IgG from which its heavy chain is derived. Figure 3b shows that, within the limitations of the measurements, the foregoing predictions hold. The spectra of the two hybrids, H^DL^N and H^NL^D, fall between the spectra of the native immunoglobulins from which their chains were derived. Also, the features of the hybrid recombinants more closely resemble the spectrum of the immunoglobulin from which their heavy chain is derived. This fact is most clearly seen by comparing the ratio of $[m']_{230}$ to $[m']_{225}$ obtained for each protein, since such comparison does not require precise knowledge of the protein concentration. The ratio obtained for native anti-DNP is 0.86 as compared to 0.89 for the H^DL^N recombinant while the ratio is 1.0 for both nonspecific IgG and the H^NL^D recombinant. Thus, the recombination of chains of quite different specificities results in molecules which are by a number of criteria, including ORD, structurally competent immunoglobulins. This supports the conclusions of Björk and Tanford (1971) which were based on similar evidence. Stevenson and Dorrington (1970) on the other hand have presented evidence that the pairing of the original heavy and light chains is required to generate a native ORD spectrum. The reason for this discrepancy in results is not clear.

Hapten Binding by Immunoglobulin Recombinants. Equilibrium dialysis results obtained for the binding of [^{3}H]DNP-lysine to the homologous recombinants are shown in Figure 4. Experimental binding ratios of 1.6 moles of hapten/150,000 g of protein were observed for H^DL^D recombined by either method at the highest free ligand concentration measured. The number of sites obtained by extrapolation of the curve in Figure 4 to the abscissa is close to 2. The affinity of both preparations was three orders of magnitude lower than that

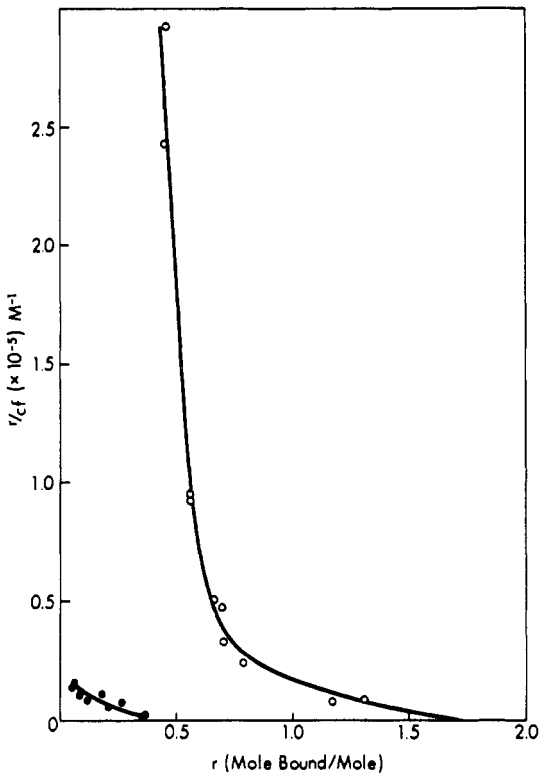

FIGURE 5: Scatchard plot for the binding of [^{3}H]DNP-lysine to heterologous recombinants of anti-DNP and nonspecific IgG H and L chains at 5°. H^DL^N at 1.31×10^{-5} M (⊙—⊙) and H^NL^D at 2.75×10^{-5} M (●—●). Data corrected for binding by nonspecific IgG. Solvent: 0.02 M sodium acetate (pH 5.5)–0.1 M NaCl.

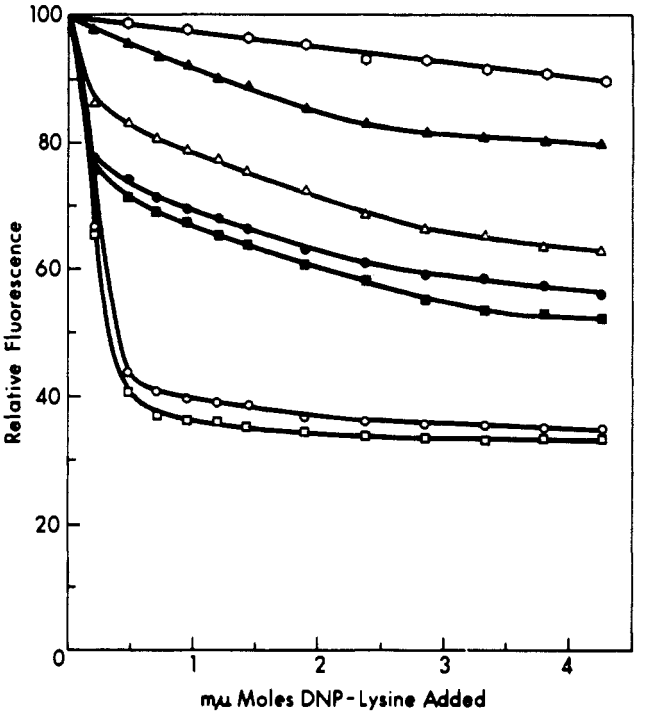

FIGURE 6: Fluorescence quenching titrations of rabbit immunoglobulin recombinants with DNP-lysine at 5° in 0.02 M sodium acetate (pH 5.5)–0.1 M NaCl. Protein concentrations, 50 μg/ml; 1.0 ml. Protein solution titrated with 2.38×10^{-5} M DNP-lysine. Excitation at 290 nm; emission at 345 nm. Mildly reduced and alkylated anti-DNP (⊙—⊙); native anti-DNP renatured from 1 M propionic acid, disulfide bonds intact (⊡—⊡); homologous anti-DNP recombinants H^DL^D renatured by method I (■—■) and method II (●—●); heterologous recombinants of anti-DNP and nonspecific IgG, H^DL^N (△—△) and H^NL^D (▲—▲); and nonspecific rabbit IgG (○—○).

of the intact molecule ($\bar{K}_0 = 3.3 \times 10^8$ M^{-1}), with $\bar{K}_0 = 1.3 \times 10^6$ M^{-1} for H^DL^D prepared by method I or II. The $\bar{K}_0$ of H^DL^D, however, was still significantly higher than that of isolated heavy chain dimer ($\bar{K}_0 = 6 \times 10^4$ M^{-1}; Painter *et al.*, 1972) or that of the heterologous recombinant, H^DL^N (see below).

The binding of [^{3}H]DNP-lysine to the heterologous recombinants, H^DL^N and H^NL^D, is shown in Figure 5. All data have been corrected for a small amount of binding by nonspecific IgG ($r = 0.31$ at the highest free ligand concentration employed). Both H^DL^N and H^NL^D showed significant hapten binding activity. H^DL^N bound 1.3–1.4 moles of hapten/150,000 g of H^DL^N at the highest free hapten concentration used (1.57×10^{-4} M). The number of sites obtained by extrapolation to infinite hapten concentration is in reasonable agreement with the expected value of 2 sites/molecule. The calculated value for $\bar{K}_0$, assuming $n = 2$ sites/mole, is 1.9×10^4 M^{-1}. A slightly higher $\bar{K}_0$ of 3.6×10^4 M^{-1} is obtained if $n = 1.6$. These $\bar{K}_0$ values are actually slightly below the value of 6×10^4 M^{-1} obtained for free heavy chain (Painter *et al.*, 1972). This result indicates that the addition of nonspecific light chain, which leads to considerable conformational rearrangement, has a minimal effect on the affinity of the specific heavy chain for hapten.

H^NL^D, in contrast to H^DL^N, has a very low, but real, binding activity. Like the light-chain binding studies described previously, an accurate estimate of $\bar{K}_0$ was difficult because of the intrinsically low affinities of these proteins. Even if the maximum number of sites was only 0.5 per mole (approximately the maximum binding achieved experimentally), the value of $\bar{K}_0$ calculated from the data in Figure 5 is no more than 5000 M^{-1}. This value represents an upper limit since it is very probable that only the higher avidity sites would be saturated under the experimental conditions used. If one assumes that $n = 2$, a lower value of $\bar{K}_0$ is obtained, which is similar to the value of 200–500 M^{-1} obtained with free anti-DNP light chain (Painter *et al.*, 1972). These admittedly weak data suggest that the presence of nonspecific heavy chain in the recombinant had little, if any, effect on the binding of hapten by the anti-DNP light chains.

The results of fluorescence quenching titrations, shown in Figure 6, are similar to the equilibrium dialysis results. Assuming that maximum quenching (quenching when all sites are filled) is the same for all species, the relative order of hapten affinities is: native = protein renatured from 1 M propionic acid (S–S intact) $\gg$ H^DL^D > H^DL^N $\gg$ H^NL^D > nonspecific IgG. One other important point may be drawn from these data, namely, full hapten binding affinity is regained (in the case of heterogeneous antibody preparations) if the interchain disulfide bonds are intact during the denaturation and renaturation processes. This control indicates that the method of denaturation and renaturation itself is not responsible for the loss of binding affinity observed in the other cases above.

The results of all hapten binding results are summarized in Table III. The lowered affinities of homologous recombinants of heavy and light chains derived from heterogeneous antibody preparations seen here have been observed in varying degrees by previous workers (Edelman *et al.*, 1963; Roholt *et al.*, 1964, 1965a,b; Haber and Richards, 1966; Lamm *et al.*,

TABLE III: Summary of Data for the Binding of [^{3}H]DNP-lysine to Reconstituted Immunoglobulins.[a]

Species	Method of Renaturation[b]	$\bar{K}_0$ (M^{-1}) × 10^{-5}	$-\Delta\bar{G}_u°$ (kcal/mole)
Mildly reduced and alkylated anti-DNP	I	3000	13.0
Renatured native anti-DNP (S–S intact)	II	≥2000[c]	≥12.8
H^DL^D	II	1.3[d]	8.73
H^DL^D	I	1.3[d]	8.73
H^DL^N	I	0.19–0.36[e]	7.7–8.1
H^NL^D	I	<0.05	<6.9

[a] Corrected for binding of hapten by reconstituted nonspecific IgG. [b] See text for details. [c] By fluorescence quenching; all others by dialysis equilibrium. [d] $\bar{K}_0$ calculated assuming a maximum of 2 binding sites/150,000 g. [e] The low value assumes $n = 2$ while the higher value is for $n = 1.6$ (see text for details).

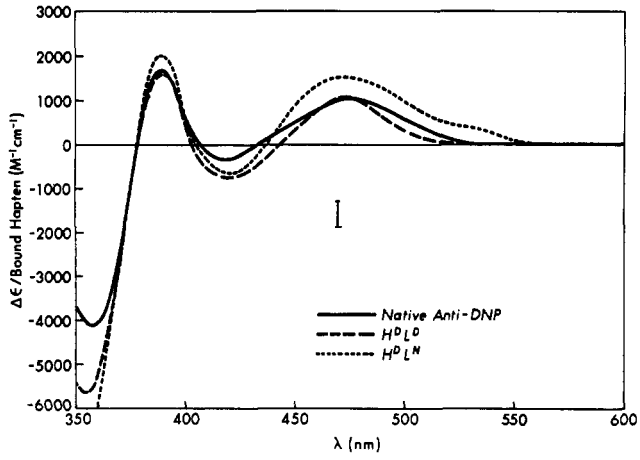

FIGURE 7: Molar difference spectra between free and recombinant-bound DNP-lysine at 5°. Native anti-DNP at 2 × 10^{-5} M (—) protein concentration; H^DL^D recombined by method I at 0.84 × 10^{-5} M (- - -); H^DL^N at 0.98 × 10^{-5} M (···). Total DNP-lysine concentration 1.90 × 10^{-5} M in 0.02 M sodium acetate (pH 5.5)–0.1 M NaCl.

1966; Hong and Nisonoff, 1966; Zappacosta and Nisonoff, 1968). These results, together with the ORD results, suggest that while all heavy- and light-chain combinations lead to the generation of apparently full structural integrity, a specific combination of heavy and light chains is required for regeneration of the original antigen binding affinity. This is more clearly demonstrated by the recent findings of Bridges and Little (1971) with homogeneous mouse myeloma proteins having anti-DNP activity. When the autologous chains from a single such protein were separated and then recombined, a complete recovery of hapten binding affinity resulted. The combination of the heavy (or light) chains of such a myeloma protein with the light (or heavy) chain from another nonbinding myeloma protein led to little or no affinity.[4] Significantly, a heterologous recombination of chains from two different myeloma proteins, both of which had anti-DNP activity, led to a loss of affinity.

Hapten Difference Spectra. In the preceding paper it was shown that the difference spectra of DNP-lysine bound to anti-DNP H_2 and to native anti-DNP are similar. It was of interest to see how the reconstituted antibody and especially, the hybrid recombinants, behave in this regard. Figure 7 shows the hapten difference spectra obtained with H^DL^D and H^DL^N. The spectra have been expressed in terms of the difference in extinction coefficients of bound and free hapten. The total DNP-lysine concentration was 1.9 × 10^{-5} M and the protein concentrations ranged from 0.85 × 10^{-5} to 1.1 × 10^{-5} M. The molar concentration of bound hapten has been calculated from the equilibrium dialysis binding data above.

[4] The apparent lack of binding affinity by heterologous recombinants of anti-DNP and nonspecific IgG H and L chains observed by these workers and a few others is probably due to the lower affinity of their starting antibody preparations as compared to ours. In the case of Bridges and Little, for example, one would calculate a K_0 of 250 M^{-1} for the isolated H chain derived from MOPC-315 (K_0 = 4.9 × 10^6 M^{-1} originally) assuming its L chain had an affinity comparable to ours.

The spectra for H^DL^D and H^DL^N show all the major features of the native antibody. No significant spectral perturbation was observed with H^NL^D under the conditions employed in Figure 7. However, under these conditions, no significant binding of hapten by H^NL^D would be expected because of its intrinsically low affinity. As discussed in the preceding paper, the observed spectral red shifts are characteristic of anti-DNP antibodies and implicate the presence of tryptophan at the binding site. These results along with the previous findings indicate that these tryptophan residues reside primarily on the heavy chain.

Discussion

Regain of IgG Structural Parameters. A number of workers have shown that the gross structural features of IgG, such as $s_{20,w}$, chain composition, and molecular weight, are regained by recombination of IgG chains at pH 5.5 (Björk and Tanford 1971; Stevenson and Dorrington, 1970) as well as by recombination in propionic acid, followed by dialysis to neutrality (Edelman *et al.*, 1963; Metzger and Mannik, 1964; Bridges and Little, 1971). This is true even if the H and L chains are derived from two different animal species (Fougereau *et al.*, 1964). The same result has been found here. The per cent yield of reconstituted anti-DNP and nonspecific immunoglobulins was nearly quantitative and was not dependent on the source of the chains used for recombination. Thus, even heterologous recombinants of anti-DNP and nonspecific IgG resulted in very high yields (75–80%). The physical and chemical characteristics, such as sedimentation behavior and the chain composition, of all recombinants examined are identical with those expected for native IgG. This indicates that the formation of a stable IgG four-chain molecule does not require pairing of the H and L chains which were associated in the original native state. In addition, the similarity of the ORD spectra of the recombined H^DL^D molecules to that of the original anti-DNP provides evidence that the internal folding of the recombined chains is identical (within the limits of detection inherent to ORD) with that of the native proteins. The spectra obtained for the hybrid recombinants are of particular interest because the populations of parent mole-

cules in this case had slightly different average ORD curves. Both H^DL^N and H^NL^D were found to possess spectra intermediate between those of the parent native proteins, such as would have been expected for hybrid recombination without structural change.

It should be recalled in this connection (Painter *et al.*, 1972) that the separated H and L chains, under native conditions, have ORD spectra that do not, when added in proper proportion, agree with the experimental ORD spectra of native or reconstituted IgG. Thus one or both chains undergo a significant conformational change upon recombination. It is clear that this conformational change occurs nonspecifically: within the limits of the measurements the conformations of heavy and light chains in any recombinant are the same as in the native molecules from which they were derived. The simplest explanation is that the ORD change that accompanies the reaction $H_2 + L_2$ (or 2L) $\rightarrow H_2L_2$ is associated with the constant regions of the polypeptide chains and thus unaffected by scrambling of chain partners.

Hapten binding free energies ($\Delta\bar{G}_u^\circ$) provide additional evidence on this point. It was shown previously that the $\Delta\bar{G}_u^\circ$ of hapten binding found for H_2 and L_2 accounted for the original $\Delta\bar{G}_u^\circ$ of the native antibody despite the conformational differences (as judged by ORD) between the free chains and intact antibody (Painter *et al.*, 1972). Furthermore, the $\Delta\bar{G}_u^\circ$ values for the hybrid recombinants, H^DL^N and H^NL^D, are quite comparable to those of the H dimer and L dimer, respectively. This argues that the binding site regions of the chains have not undergone any conformational change which is sufficient to alter hapten binding energies. Since hapten binding energy provides the most sensitive criterion available for assessing the structural integrity of a combining site, it appears that the internal conformations of the individual binding regions donated by each chain in the recombinant molecules remain intact, independent of the manner of association with other chains and conformational differences resulting therefrom.

Several workers have obtained experimental evidence suggesting, in contrast to the conclusions of this paper, that specificity is involved in the physical combination of H and L chains (Grey and Mannik, 1965; Mannik, 1967). Although it was found that any pair of H and L chains (from human myeloma proteins) could form a stable IgG molecule, it was observed that, in the presence of a large excess of a heterologous L chain, a particular H chain combined with its autologous L chain to a greater degree than would be expected if recombination was a random process. However, the phenomenon was variable from one myeloma protein to the next. In many cases, nonspecific L chains competed for a myeloma H chain almost as efficiently as the autologous L chain. Such specificity may not necessarily be related to antibody binding specificity, and may be due to allotypic variations between myeloma proteins rather than idiotypic ones. The recently discovered genetic subclasses that exist in the variable regions of H and L chains might also be involved (Hood and Ein, 1968; Edelman *et al.*, 1969).

Hapten Binding by the Heterologous Recombinants. Two significant conclusions can be drawn from the data for the binding of DNP-lysine to the heterologous recombinants made between the chains derived from anti-DNP and nonspecific IgG.

(1) The number of binding sites observed for the H^DL^N recombinant was at least 1.5 per 150,000 molecular weight, approaching 1 site per H chain, whereas the corresponding experiments with isolated pure H chain yield only 1 binding site/two H chains. This result is consistent with the model presented previously for the structure of H chain dimer (Painter *et al.*, 1972), where the 2 binding site regions of each *H* chain are brought together in the *H* dimer in such a way as to form a single hapten-accessible site per dimer. It is reasonable to expect that both H chain sites would become equally accessible to hapten after reaction with L chain since the two resulting Fab regions are essentially independent of one another (Noelken *et al.*, 1965; Valentine and Green, 1967; Steiner and Lowey, 1966). This strongly supports the previous conclusion that the finding of one binding site per H dimer was not an accidental one.

(2) The similarity of the average binding constants ($\bar{K}_0$) for isolated H dimer and H^DL^N, as discussed previously, provide strong evidence that L chain exerts no "allosteric" control on the H chain binding region. The same kind of conclusions may be drawn from the H^NL^D binding result but not with the same degree of certainty, because of the technical inability to saturate all available binding sites.

Hapten Binding by the Homologous Recombinants. The combination of H and L chains derived from the same antibody preparation has usually led to an enhancement of $\bar{K}_0$ relative to the case where specific H and nonspecific L are combined. With the exception of the homogeneous myeloma proteins mentioned above, no such recombinant has regained full affinity for antigen (Roholt *et al.*, 1965a,b; Haber and Richards, 1966; Hong and Nisonoff, 1966; Lamm *et al.*, 1966; Zappacosta and Nisonoff, 1968). Our results generally agree with these previous reports, confirming that the combination of specific H and specific L chains leads to a higher affinity for hapten than does a heterologous recombinant. The data of Haber and Richards (1966), which were obtained with rabbit anti-DNP preparations having an affinity and a degree of heterogeneity similar to our preparation, revealed a much larger difference in $\bar{K}_0$ between the homologous and heterologous recombinants than our data indicate. The difference between our results and those of Haber and Richards may be explained by the fact that the results of the latter authors were based on fluorescence quenching titrations made at very low free hapten concentrations. As a result, they probably reflect only those recombinant molecules with the highest affinities whereas our data represent the average binding constants for the entire population.

Although it seems probable that most, if not all, H- and L-chain combinations lead to structurally intact immunoglobulins, hapten binding affinity is fully recovered only when the H and L chains which were initially paired with one another are brought together. The generation of an antibody site which is exactly complementary to the original site requires that *autologous* H and L chains, such as those of myeloma proteins with binding activity, be combined (Bridges and Little, 1971). Alternatively, antibodies produced by clones of uniform cells can be used (Klinman, 1971).

These results, together with the evidence for structural integrity of the binding regions, and for the simple additivity of the free energies of binding, indicate that in a heterogeneous mixture of antibodies the apportionment of affinity for the hapten is distributed in different ways between H and L chains, so that a light chain which uniquely complements the heavy chain with which it was originally associated may enhance the affinity of some other specific H chain to only a small extent or not at all.

It is possible that a pair of H and L chains, mismatched for optimal binding of DNP-lysine, may have very high affinity for a related antigen possessing similar reactive groups with a

different spatial arrangement. The results we have obtained are consistent with the possibility that a relatively large number of antibody specificities could be generated by different ways of combining relatively few distinct heavy chains with relatively few distinct light chains, as was suggested in the previous paper (Painter *et al.*, 1972). We know of no evidence to indicate whether or not such a process is important in the natural generation of antibody specificity.

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Inhibition of Ribonucleic Acid Synthesis by Myxin†

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ABSTRACT: The inhibition of DNA template-controlled RNA synthesis has been studied kinetically in the absence and presence of the antibiotic myxin. The rates as function of concentration of the four ribonucleotides, one varied at a time, have been measured and could be expressed in a Michaelis–Menten equation. Myxin inhibits the RNA chain growth by affecting the rate terms for CTP and GTP, but not for ATP and UTP. The control of myxin on the inhibition of incorporation of C and G is equally effective. The base-specific behavior of myxin is compared to that of other antibiotics.

The antibiotic myxin, isolated from a *Sorangium* species, has been reported to possess an unusually broad antimicrobial spectrum. It is capable of inhibiting growth of a wide variety of microorganisms including gram-positive and -negative bacteria, fungi, actinomycetes, and yeasts (Peterson *et al.*, 1966). Myxin has been synthesized following several unambiguous and independent routes allowing the assignment of its structure 1-hydroxy-6-methoxyphenazine 5,10-dioxide (Sigg and Toth, 1967; Weigele and Leimgruber, 1967). The *in vivo* effect of the antibiotic has been found to consist of an inhibition of DNA synthesis and RNA synthesis, and a reduction of protein synthesis. It was speculated that the activity of myxin was due to interaction with the DNA-polymerizing enzyme without being bound to DNA (Lesley and Behki, 1967). Recently we have found that phenazine antibiotics, including myxin, inhibit the *in vitro* RNA synthesis and that these compounds bind to DNA. Therefore, we concluded that the association of antibiotic with DNA, presumably through intercalation, is a contributory factor, if not the sole cause, in the inhibition of DNA tem-

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