RECOMBINATION OF ANTIBODY POLYPEPTIDE CHAINS IN THE PRESENCE OF ANTIGEN

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Several recent studies have suggested that the partially reduced, alkylated, and acid-dissociated polypeptide chains of gamma$_2$ globulins can reassociate in an orderly manner yielding molecules with antibody activity greater than that found in the isolated subunits (1–3). Lack of specificity in reassociation was demonstrated in that the antibody H chains[1] readily reassociated with L chains derived from non-specific gamma$_2$ globulin and *vice versa* (3). Other experiments, however, clearly indicate that the interaction of antibody H and L chains is influenced by the presence of the specific antigen, since in the presence of the latter, the polypeptide chains are not as readily separable as in the absence of the antigen (8).

The present studies were undertaken to evaluate the recombination of the polypeptide chains of an antihapten antibody in the presence of a specific hapten. Our observations indicate that the presence of the hapten induces a selective recombination between the antibody subunits. Moreover, the activity of the recombined antibodies is enhanced when the recombination proceeds in the presence of the hapten.

Materials and Methods

Rabbit Antibodies and Gamma$_2$ Globulin.—Rabbit antibodies directed to the 2,4-dinitrophenyl determinant (anti-DNP) were prepared and purified according to the procedure of Farah *et al.* (9). Approximately 90 per cent of the isolated antibody protein was precipitable by the DNP-bovine gamma$_2$ globulin (DNP-BγG) antigen.

Non-specific rabbit gamma$_2$ globulin was prepared from rabbit anti-DNP antiserum from which all of the anti-DNP had been specifically precipitated. The supernatant serum was rendered 40 per cent saturated with ammonium sulfate; the salted out protein was redissolved and dialyzed against 0.0175 M sodium phosphate buffer, pH 6.5. It was then further purified by DEAE cellulose chromatography (10).

[1] The procedures used for the separation of polypeptide chains in this paper are those recommended by Fleischman *et al.* (4). It is now abundantly clear that the polypeptide chains derived by partial reduction, followed by dissociation in 1 M propionic acid, termed A and B chains by the above investigators, correspond to the preparations obtained on full reduction and separation in urea (5, 6) and respectively termed heavy (H) and light (L) chains (7). The latter terminology has a useful mnemonic value.

Iodination of Proteins.—The isolated antibodies and gamma$_2$ globulins were labeled with I^{131} (carrier-free I^{131} was obtained from the Abbott Laboratories, Oak Ridge, Tennessee) or with I^{125} (carrier-free I^{125} was obtained from Nuclear Science and Engineering Corporation, Pittsburgh) according to the procedure of Helmkamp *et al.* (11). Proteins were labeled with 1 to 2 moles of iodine per mole of protein. Unbound iodine was removed by passage over columns of dowex 1X4 in chloride phase. The final preparations contained less than 0.1 per cent of unbound radioactivity.

Reduction, Alkylation, and Separation of H and L Chains.—Disulfide bonds of the isolated proteins were reduced with 0.2 M 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, New York) and the resulting sulfhydryl groups were alkylated with iodoacetamide (thrice recrystallized from water) in a pH 8.2 tris-HCl buffer, according to the procedure of Fleischman *et al.* (4). Following dialysis against 1 M propionic acid, the reduced and alkylated materials were separated into the polypeptide chains according to the procedure of the latter authors, except that columns of sephadex G-100 (70 x 3 cm) equilibrated with 1 M propionic acid were used. Flow rates were approximately 15 ml per hour. Appropriate fractions of the effluent were pooled and concentrated in Visking membranes (wall thickness 0.0020 inches) at 10 to 12 pounds positive pressure at 4°C. Concentration was allowed to proceed until the protein concentration was 1 to 2 mg per ml.

Procedure for Recombination.—Aliquots of appropriate pools of isolated H or L chains were mixed and then subjected to a scheme of dialysis that yielded essentially total recovery of the constituents without any loss due to precipitation. Each step of dialysis was carried out for 12 to 18 hours in thin walled (0.0008 inches) Visking dialysis membranes using a 100-fold excess of outer solvent at a temperature of 4°C. The steps of dialysis were: (*a*) 1.0 M propionic acid, once, (*b*) 0.01 M sodium acetate buffer, pH 5.0, two times, and (*c*) 0.01 M sodium acetate buffer pH 5.0 in 0.15 M NaCl, two times. In experiments involving hapten, *N*-2,4-dinitrophenyl-ε-aminocaproic acid (DNP-aminocaproate), prepared by the method of Carsten and Eisen (12), was added to the outer solvent to a final concentration of 3×10^{-5} M.

Density Gradient Ultracentrifugation.—Aliquots of the dialyzed recombined materials were subjected to sucrose density gradient ultracentrifugation (13) to separate the free L chains, recombined 7S molecules, and possible heavier aggregates. For this purpose gradients of 5 to 20 per cent sucrose or 10 to 30 per cent sucrose in 0.01 M sodium acetate buffer, pH 5.0 in 0.15 M NaCl, were constructed. The gradient volume was 4.90 ml, and a 0.10 ml aliquot of the specimen to be analyzed was applied to the gradient. Following an 18 hour run at 4°C at 36,000 RPM in the Spinco model L centrifuge, the gradients were harvested through a puncture hole in the bottom of the tube. I^{131} and I^{125} were counted in each fraction and in the bottom of the tube. The results were expressed as counts per unit time and were plotted against per cent volume of gradient, 100 per cent being 5.00 ml and representing the top of the sucrose gradient. The localization of the 7S material and of the free L chains was highly reproducible from experiment to experiment.

Activity Measurements.—Antibody activity was assayed by two methods:

1. Uniodinated pooled rabbit anti-DNP was added to an aliquot of the iodinated sample to be assayed. A standard precipitin curve was then constructed using the DNP–bovine gamma$_2$ globulin as antigen. The thrice-washed precipitates were assayed for I^{125} and I^{131}.

2. To duplicate 0.2 ml aliquots of the iodinated sample to be assayed (containing approximately 3×10^{-6} millimoles protein) was added 0.10 ml 0.01 M sodium phosphate buffer pH 7.5 in 0.15 M NaCl with or without 1.5×10^{-3} millimoles DNP-aminocaproate. Subsequently 3×10^{-6} millimoles of DNP-bovine gamma$_2$ globulin (equivalent to about 2×10^{-4} millimoles dinitrophenyl groups) in 0.1 ml was added. Following thorough mixing, 2 ml of a 5×10^{-2} M streptomycin sulfate solution was added. The resulting precipitates (9) were washed twice with 5×10^{-2} M streptomycin with or without 8×10^{-5} M DNP-aminocaproate. The washed precipitates were assayed for I^{125} and I^{131}. Control tubes which had omitted from them one or more of the ingredients were included.

Miscellaneous.—All chemicals used were reagent grade, unless otherwise specified. Glass-distilled water was used throughout the experiments. After the initial reduction of the isolated non-specific gamma$_2$ globulin and antibodies all procedures were carried out at 4°C. Protein concentrations were determined by the modified Folin-Ciocalteau phenol method (14).

The counting of I^{131} and I^{125} was performed in a Packard automatic well type scintillation counter. Since all the data are presented in the form of percentage of total activity no corrections for radioactive decay were made.

RESULTS

Recombination of H and L Chains from Rabbit Gamma$_2$ Globulin.—Reduced and alkylated gamma$_2$ globulin yielded two major peaks on elution from columns of sephadex G-100 equilibrated with 1 M propionic acid similar to the patterns originally obtained by Fleischman *et al.* (4). The G-100 fractionation, however, led to partial splitting of the H chain peak (Fig. 1). The L chains were very well separated from the H chain peaks. On the basis of the Folin protein determination the yield of L chains averaged 33 per cent of the total protein eluted from the column. The specific radioactivity was approximately equivalent for the three protein peaks indicating relatively uniform iodination of the H and L chains.

Initial experiments were conducted to study the influence of the conditions of dialysis for recombination on the isolated H and L chains. Under the conditions described above (Materials and Methods), the L chains remained completely soluble and gave a symmetrical single peak on sucrose density gradient ultracentrifugation, as illustrated in Fig. 2. On similar treatment the isolated H chains showed a variable amount of flocculation, and only about 35 per cent of the protein remained in solution. Upon density gradient ultracentrifugation of the soluble material aggregation of H chains was evident as illustrated in Fig. 2.

Subsequently, I^{131}-labeled L chains were mixed with a threefold molar excess of I^{125}-labeled H chains.[2] Following the procedure outlined for recombination, no gross precipitation of material was evident. Density gradient ultracentrifugation was performed and the distribution of L chains, recombined 7S material, and heavier aggregates is illustrated in Fig. 3. It is clear that all the L chains were combined with the H chains, and that some of the excess H chains formed heavier aggregates.

A number of experiments were conducted in which I^{125}-labeled H chains were recombined with a twofold molar excess of I^{131}-labeled L chains. Again, essentially total recovery of the labeled material was obtained in soluble form. On density gradient ultracentrifugation the recombined materials were separated from the excess L chains. Sixty to seventy per cent of the H chains were found in the 7S peak. The remainder of the labeled H chains were found in heavier aggregates or on the bottom of the centrifugation tube. Analysis of the label on the L chains also indicated that 60 to 70 per cent of the expected material

[2] Gamma$_2$ globulins contain H and L chains in a molar ratio of 1:1 (15–17).

was recombined. Only a small percentage of the label of the L chains was found in the material heavier than 7S.

On separation of the H and L chains on columns of sephadex G-100 a bimodal peak of H chains was obtained, as already illustrated in Fig. 1. It is thought

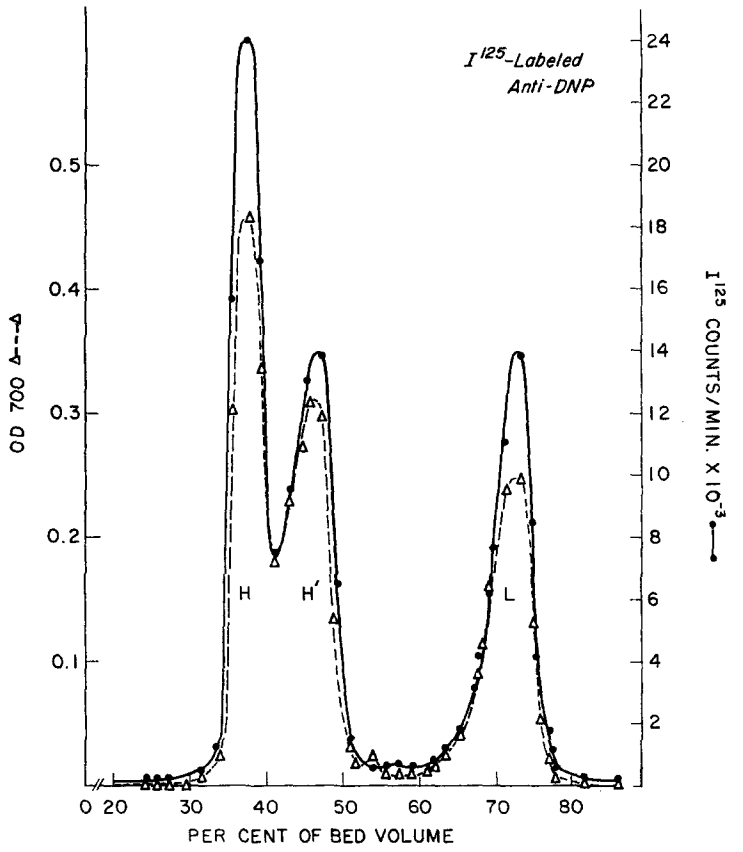

FIG. 1. Sephadex G-100 fractionation of 65 mg I^{125}-labeled, reduced, and alkylated anti-DNP. Solvent: 1 M propionic acid. Sephadex bed dimensions: 70 x 3.0 cm. Flow rate: 15 ml per hour. Left ordinate: Folin OD. Right ordinate: I^{125} counts.

that the first peak (*H*) represents dimers or heavier aggregates of H chains, and that the second peak (*H'*) is constituted of H chain monomers. Experiments were conducted to study the recombination of these individual H chain peaks with L chains. Again, the H chains were obtained from I^{125}-labeled and the L chains from I^{131}-labeled gamma$_2$ globulins. Recombination experiments were set up using a twofold molar excess of L chains. With the first peak of H chains (*H*), 33 per cent of the I^{125} label (H chains) and 9 per cent of the I^{131} label (L

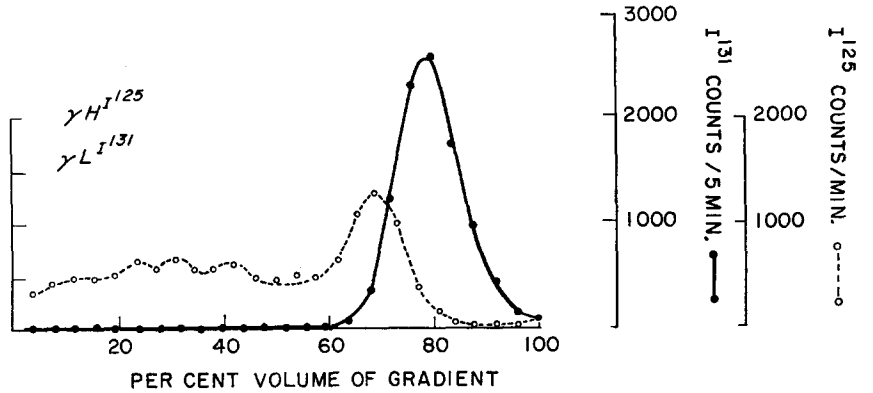

FIG. 2. Sucrose density gradient ultracentrifugation pattern of isolated L chains labeled with I^{131} and H chains labeled with I^{125} subsequent to dialysis. Data of separate experiments are superimposed for comparison of distribution of L chains and H chains. Gradients of 10 to 30 per cent sucrose were utilized. In this and all subsequent illustrations of density gradient experiments the top of the gradient is represented by 100 per cent volume of the gradient.

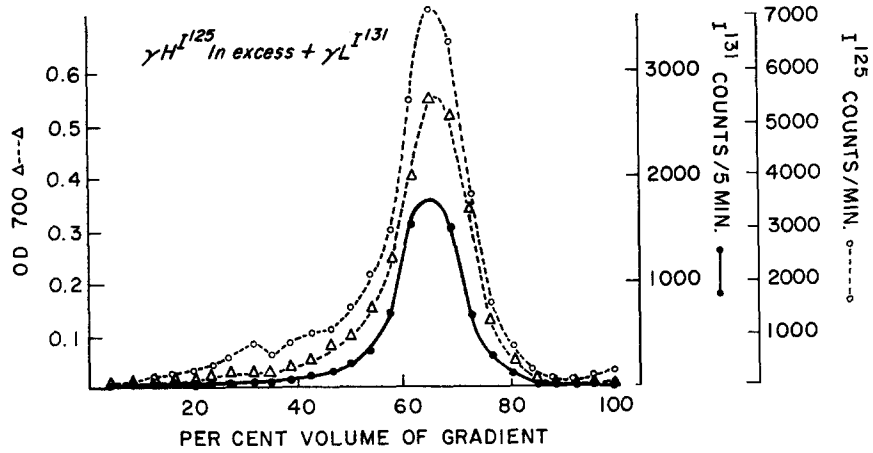

FIG. 3. Sucrose density gradient ultracentrifugation pattern of a mixture of I^{131}-labeled L chains with a threefold molar excess of I^{125}-labeled H chains. Gradient of 10 to 30 per cent sucrose. Left ordinate: Folin OD.

chains) was found in the material sedimenting faster than the 7S peak (Fig. 4 *a* and Table I). In contrast, when the second peak of H chains (*H'*) was used for recombination, only 1 per cent of both I^{125}- and I^{131}-labeled material sedimented faster than the 7S peak (Fig. 4 *b* and Table I).

Once recombination of the H and L chains has occurred, the pH and ionic strength can be further increased without aggregation and loss of protein. A

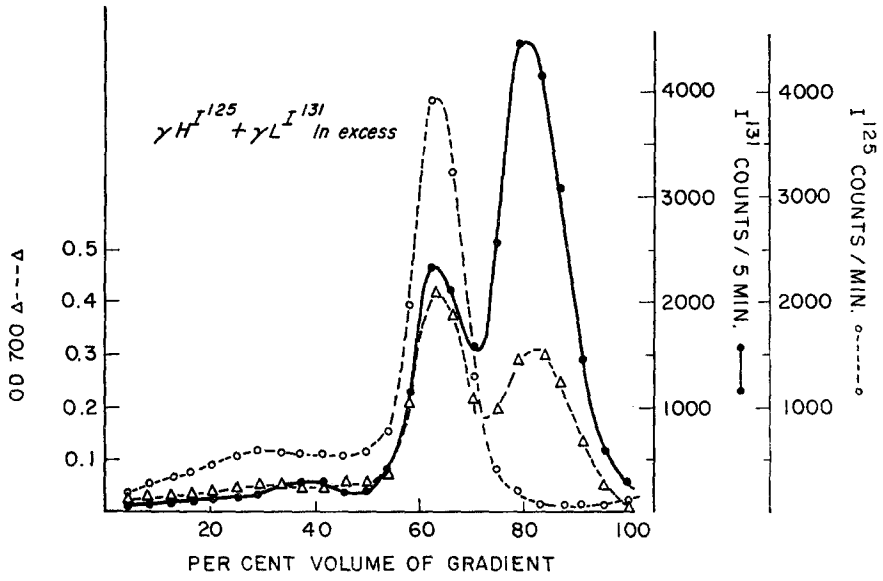

FIG. 4 *a*

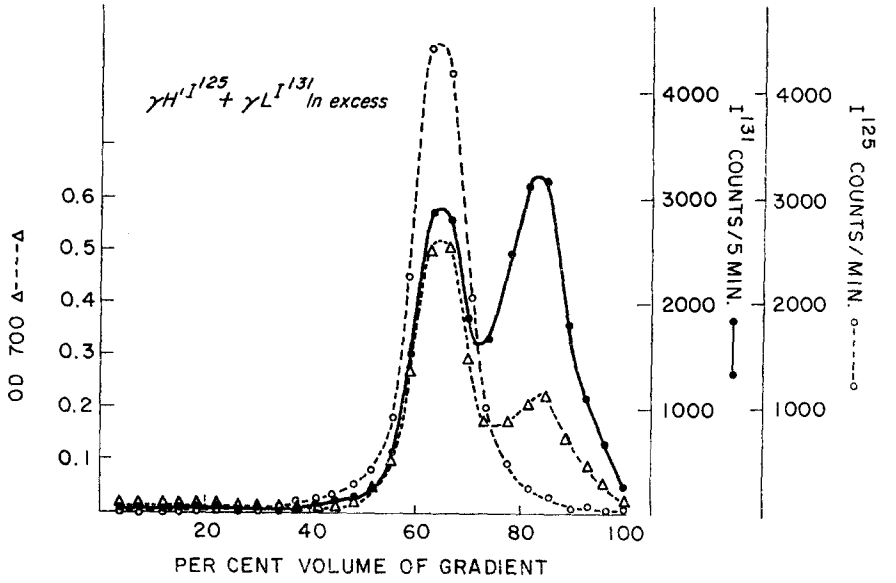

FIG. 4 *b*

FIGS. 4 *a* and 4 *b*. Sucrose density gradient ultracentrifugation pattern. Gradient of 10 to 30 per cent sucrose. Left ordinate: Folin OD.

FIG. 4 *a*. A mixture of I^{125}-labeled H chains (Fraction H, Fig. 1) with a twofold molar excess of I^{131}-labeled L chains.

FIG. 4 *b*. A mixture of I^{125}-labeled H chains (Fraction H′, Fig. 1) with a twofold molar excess of I^{131}-labeled L chains.

mixture of H and L chains that had been brought to pH 5.0 and 0.10 M NaCl and 0.01 M sodium acetate buffer was further dialyzed against 0.01 M phosphate buffer, pH 7.0, in 0.10 M NaCl. On density gradient ultracentrifugation there was no change in the distribution of the two isotopes of iodine in the 7S and the L chain peaks. Similarly there was no change in the pattern when the mixture was dialyzed against 0.2 M NaCl at pH 5.0 prior to centrifugation.

Recombination experiments were also performed without separating H and L chains on sephadex G-100. For this purpose an aliquot of gamma$_2$ globulin was labeled with I^{131}, reduced, alkylated, and dialyzed against 1 M propionic acid. To this preparation isolated L chains labeled with I^{125} were added in a quantity equal to the L chains in the unseparated mixture, thus providing a twofold excess of the L chains with respect to H chains. Subsequently, the steps of dialysis

TABLE I

Recombination of Sephadex "H" and "H'" Components with L Chains

Specimen	Density gradient fraction	I^{125}	I^{131}
		per cent	*per cent*
$\gamma H^{I^{125}}$ + excess $\gamma L^{I^{131}}$	>7S	33.4	9.0
	7S peak	62.2	25.4
	L chain peak	4.4	65.6
$\gamma H'^{I^{125}}$ + excess $\gamma L^{I^{131}}$	>7S	1.0	1.0
	7S peak	92.0	41.0
	L chain peak	7.0	58.0

Total I^{125} counts, 18,000; total I^{131} counts, 25,000.

were carried out as described above. Density gradient ultracentrifugation was performed and the two isotopes were analyzed in the gradient fractions as illustrated in Fig. 5. As previously discussed, the H and L chains were equivalently iodinated, and therefore the counts due to $L^{I^{131}}$ in the 7S peak could be calculated. In the experiment shown in Fig. 5, 32 per cent of the I^{125}-labeled L chains and 30 per cent of the I^{131}-labeled L chains had recombined with the H chains. Thus clearly, random exchange of L chains can occur in 1 M propionic acid, between isolated L chains and the dissociated but unseparated chains of gamma$_2$ globulin.

As mentioned above, by Folin analysis the L chains contain 33 per cent of the total gamma$_2$ globulin protein. On analysis of the 7S peak obtained after mixing of I^{125}-labeled H chains and I^{131}-labeled L chains a very similar percentage of the total protein in the peak was represented by the L chains. Therefore, recombination of the chains leads to a species with an H:L chain ratio equivalent to that found in the original globulin.

Recombination of H and L Chains in the Presence of Hapten.—It has been demonstrated that rabbit anti-DNP antibodies contain tyrosine in the combining sites (18). Therefore, preliminary experiments were carried out in which isolated antibodies were iodinated in the presence and absence of 0.1 M 2,4-dinitrophenol to assess if iodination at low levels would alter the antibody-combining activity. Unbound iodine and dinitrophenol were removed from the preparations by passage over columns of dowex 1X8. The precipitating activity of the antibodies protected with hapten during iodination did not differ from

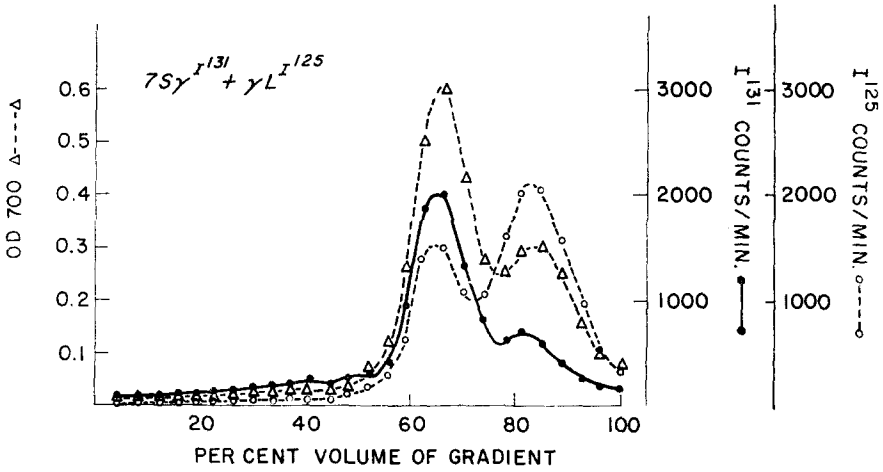

FIG. 5. Sucrose density gradient ultracentrifugation pattern of a mixture of dissociated but unseparated polypeptide chains of I^{131}-labeled gamma$_2$ globulin, and I^{125}-labeled L chains from gamma$_2$ globulin. Gradient of 10 to 30 per cent sucrose. Left ordinate: Folin OD.

that of the antibodies iodinated in the absence of the hapten. Therefore, in the subsequent experiments iodination of antibodies was carried out in the absence of hapten.

L chains were isolated from purified anti-DNP and labeled with I^{125} ($AbL^{I^{125}}$); L chains were similarly isolated from non-specific rabbit gamma$_2$ globulin labeled with I^{131} ($\gamma L^{I^{131}}$). H chains were isolated from unlabeled anti-DNP (AbH) and from unlabeled non-specific gamma$_2$ globulin respectively (γH). The labeled L chains from anti-DNP and the labeled L chains from non-specific gamma$_2$ globulin were mixed in a 1:1 ratio. The L chain mixture was then added to either a preparation of H chains from anti-DNP or to H chains from non-specific gamma$_2$ globulin. In all mixing experiments the H:L ratio was set at 1:1 on the basis of Folin optical densities (equals 1:2 on a molar basis). The mixtures were then carried through the steps of dialysis for recombination and were analyzed by density gradient ultracentrifugation.

In the absence of the hapten the H chains from anti-DNP and the H chains

from non-specific gamma$_2$ globulin did not differentiate between the L chains, except to a minor degree. As outlined in Table II, the ratio of I^{125}:I^{131} in the 7S peak was nearly the same as that in the original L chain mixture counted at the same time.

Similar experiments were repeated with hapten present throughout the dialysis procedure. H chains at a concentration of 2×10^{-5} M were combined with the mixture of labeled L chains, and then were dialyzed against 100

TABLE II

Results of Recombining H and L Chains in the Presence and Absence of DNP-Aminocaproate

Specimen	Density gradient fraction	Recombined in presence of hapten			Recombined in absence of hapten		
		I^{131}	I^{125}	I^{125}/I^{131}	I^{131}	I^{125}	I^{125}/I^{131}*
		per cent	*per cent*		*per cent*	*per cent*	
$\gamma H + AbL^{I^{125}}, \gamma L^{I^{131}}$	>7S	5.2	3.5	1.5	4.3	3.2	1.9
	7S peak	39.2	33.4	1.9	38.5	30.8	2.0
	L chain peak	55.6	63.1	2.6	57.2	66.0	2.9
Total‡				2.3			2.5
$AbL^{I^{125}}, \gamma L^{I^{131}}$				2.3			2.5
$AbH + AbL^{I^{125}}, \gamma L^{I^{131}}$	>7S	2.8	4.3	3.5	4.8	5.5	1.5
	7S peak	26.6	54.0	4.7	37.6	41.5	1.9
	L chain peak	70.6	41.7	1.4	57.6	53.0	2.6
Total‡				2.3			2.3
$AbL^{I^{125}}, \gamma L^{I^{131}}$				2.3			2.3

* These specimens were counted at a somewhat later date than the others so that the I^{131} decay gave a slightly higher I^{125}/I^{131} ratio.

‡ Total I^{125} counts: 20,500 to 27,500; Total I^{131} counts: 9000 to 12,000.

volumes of propionic acid containing 3×10^{-5} M DNP-aminocaproic acid. The limited solubility at acid pH's prevented the use of higher concentrations of hapten. The same concentration of hapten was present throughout the subsequent steps of dialysis. When the mixture of the L chains from anti-DNP and non-specific gamma$_2$ globulin was recombined with the H chains from non-specific gamma$_2$ globulin in the presence of the hapten, the pattern of recombination did not differ from the experiments done in the absence of hapten (see Table II). However, when anti-DNP H chains were recombined with a mixture of I^{125}-labeled anti-DNP L chains and I^{131}-labeled non-specific L chains in the presence of hapten, the antibody H chains preferentially recombined

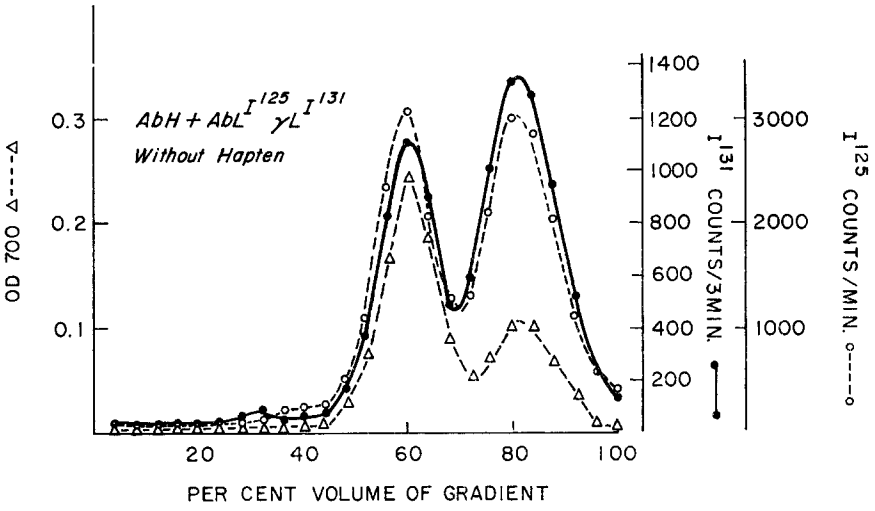

FIG. 6 *a*

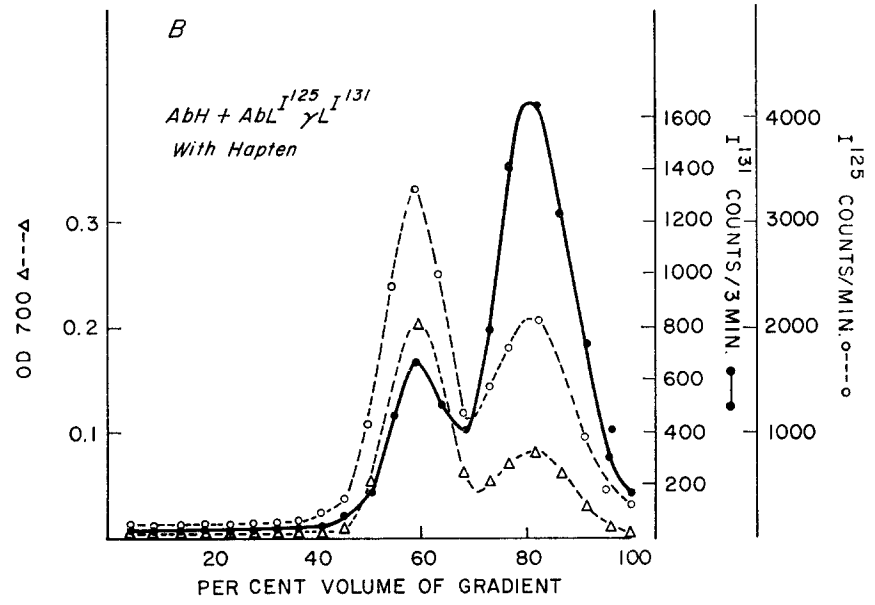

FIG. 6 *b*

FIGS. 6 *a* and 6 *b*. Sucrose density gradient ultracentrifugation pattern. Gradient of 5 to 20 per cent sucrose. Left ordinate: Folin OD.

FIG. 6 *a*. A mixture of uniodinated anti-DNP H chains with I^{125} labeled anti-DNP L chains and I^{131} labeled non-specific gamma$_2$ globulin L chains. Reassociation was performed in the absence of hapten.

FIG. 6 *b*. Same as Fig. 6 *a* except hapen (DNP-aminocaproate) was present during reassociation.

with the antibody L chains. Two-thirds of the recombined L chains were derived from the anti-DNP, in contrast to a completely random recombination in the absence of the hapten. The pertinent density gradient centrifugation patterns are illustrated in Figs. 6 *a* and 6 *b* and the data are listed in Table II.

As is indicated in Table II the ratio of I^{125} to I^{131} in the total counts recovered from the sucrose gradient is identical with the ratio of those isotopes in the original L chain mixture counted at the same time. This indicates that no preferential loss of either of the L chains occurred in any of the experiments.

TABLE III

L Chain Exchange with Dissociated Antibody Polypeptide Chains in the Absence and Presence of DNP-Aminocaproate

Specimen	Density gradient fraction	I^{125}	I^{131}	I^{125}/I^{131}
		per cent	*per cent*	
Ab + $AbL^{I^{125}}$, $\gamma L^{I^{131}}$	7S peak	14.8	11.4	1.78
No hapten added	L chain peak	85.2	88.6	1.32
Total				1.37
Ab + $AbL^{I^{125}}$, $\gamma L^{I^{131}}$	7S peak	13.5	7.0	3.14
Hapten added before L chain mixture	L chain Peak	86.5	93.0	1.52
Total				1.64
Ab + $AbL^{I^{125}}$, $\gamma L^{I^{131}}$	7S peak	18.0	8.4	3.22
Hapten added after L chain mixture	L chain peak	82.0	91.6	1.35
Total				1.50

In these experiments approximately 3 moles of iodinated L chains were added per mole of unlabeled antibody L chains. Assuming random exchange of L chains, it may be calculated that 19 per cent of the iodinated L chains should have recombined with H chains.

The total amount of recombination in these experiments, always employing an excess of L chains, was constant regardless of the H chain preparation used and was not altered by the presence of the hapten. Approximately 60 to 70 per cent recombination was achieved. The incomplete recombination may be secondary to dimerization and aggregation of H chains as described above, since in these experiments pooled H chain preparations were used, rather than the H or H′ materials illustrated in Fig. 1.

The above experiments were performed utilizing antibody and non-specific $gamma_2$ globulin from the same rabbit which was allotypically homozygous at the 1 and 4 loci[3] (19). Exactly similar results were obtained when pooled rabbit

[3] We thank Dr. S. Dray for making such rabbits available to us.

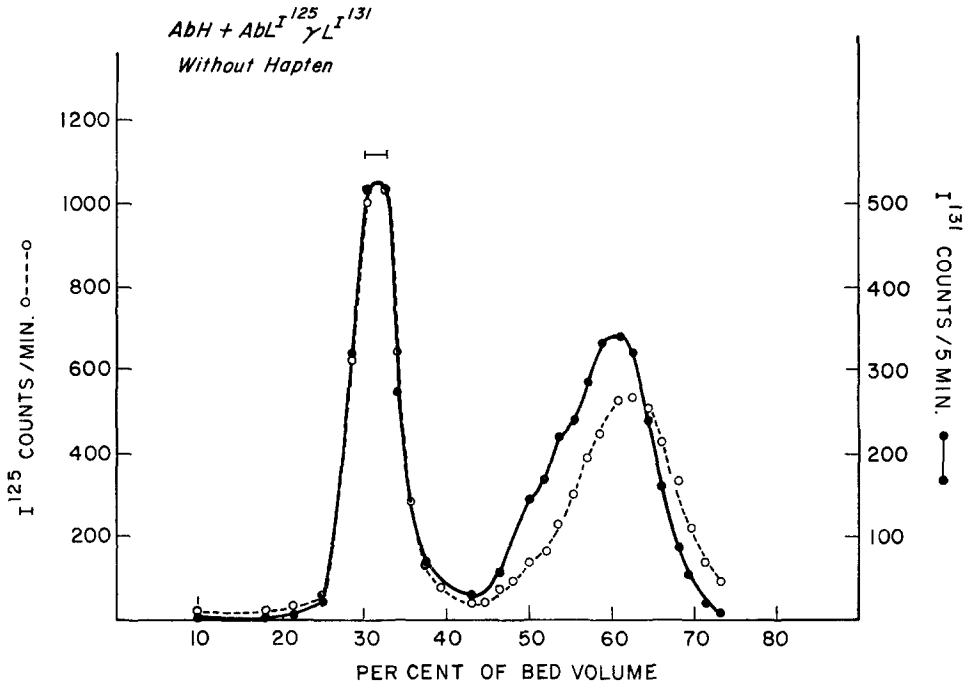

FIG. 7 *a*

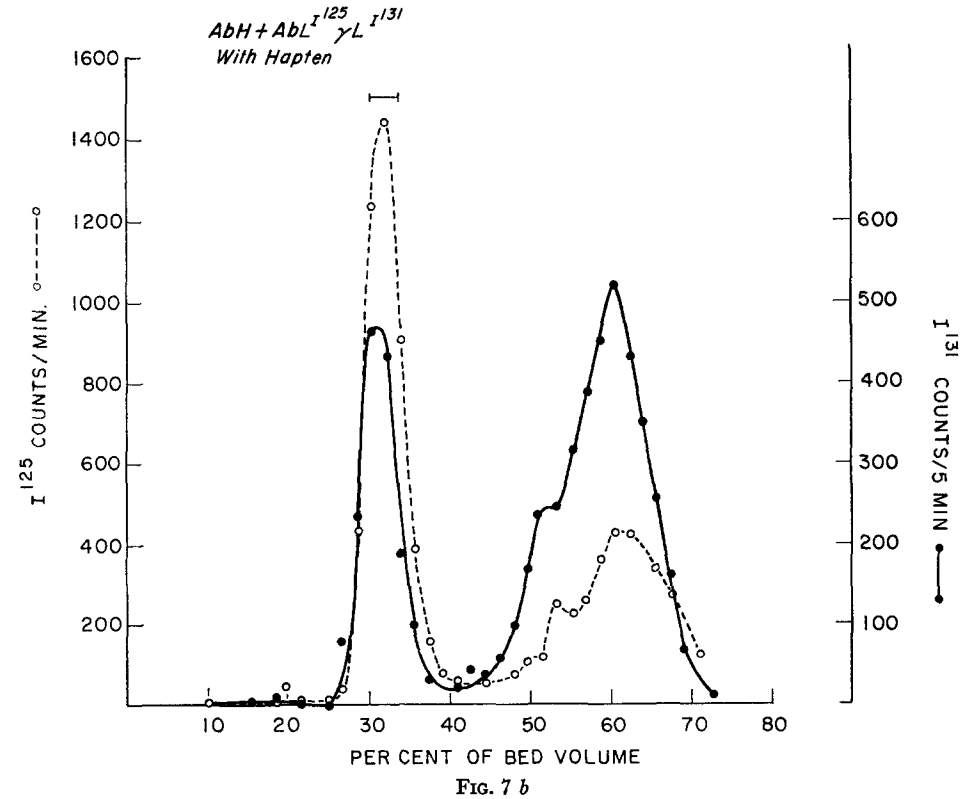

FIG. 7 *b*

FIGS. 7 *a* and 7 *b*. Sephadex G-100 fractionation of the same materials used for the density gradient ultracentrifugation illustrated in Figs. 6 *a* and 6 *b*. Sephadex bed volume 157 ml. Flow rate: 8.5 ml per hour. Protein load approximately 1.5 mg. The cross-bars indicate the tubes pooled for activity analysis (see text).

FIG. 7 *a*. Reassociation in the absence of hapten.

FIG. 7 *b*. Reassociation in the presence of hapten.

anti-DNP and pooled rabbit gamma$_2$ globulins (from unimmunized animals) were employed.

Similarly, when a mixture of I^{131}-labeled non-specific gamma$_2$ globulin L chains and I^{125}-labeled anti-DNP L chains was added to the dissociated but unseparated H and L chains of uniodinated anti-DNP, the presence of hapten caused a preferential exchange (of similar magnitude) between the antibody L chains. As may be seen from Table III, adding the hapten before or after adding the L chain mixture produced the same result. The antibody in the absence of hapten showed only a minimal preference for the antibody L chains compared to the non-specific L chains.

TABLE IV

Precipitation of H-L Recombinants in Presence of Uniodinated Anti-DNP and DNP-BγG

Recombinant specimen	Recombined in the presence of hapten		Recombined in the absence of hapten		Precipitation in absence of DNP-BγG; in presence of rabbit anti-human S fragment–human S fragment	
	I^{125} precipitated at equivalence	I^{131} precipitated at equivalence	I^{125} precipitated at equivalence	I^{131} precipitated at equivalence	I^{125} precipitated at equivalence	I^{131} precipitated at equivalence
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
AbH-Ab$L^{I^{125}}$, $\gamma L^{I^{131}}$	45.2	32.2	25.7	16.8		
γH-Ab$L^{I^{125}}$, $\gamma L^{I^{131}}$	5.9 ± 1.1*	4.4 ± 1.3*	6.0 ± 1.7*	4.6 ± 1.5*	2.3 ± 1.3*	2.7 ± 1.0*
Δ	39.3	27.8	19.7	12.2		

* Average of four tubes.

A sample of antibody H chains which had been allowed to reassociate with a mixture of non-specific and antibody L chains in the absence of hapten was run on a gradient containing hapten. The 7S peak showed a completely random distribution of L chains. Therefore, the critical time for the hapten to exert its effect was during the period of dialysis. Once the H and L chains had recombined and were at neutral pH, the L chain distribution was no longer alterable by hapten.

For the activity studies of the recombined materials to be discussed below, the recombined mixtures of the H and L chains were applied to columns of sephadex G-100 equilibrated with 0.01 M sodium acetate buffer, pH 5.0, in 0.15 M NaCl. The distribution of L chains in the recombined material and in the free L chains was exactly the same as the distribution pattern obtained on sucrose density gradient ultracentrifugation. The recombination of anti-DNP H chains with the mixture of L chains again showed a preferential recombination with the antibody L chains in the presence of hapten (Figs. 7 *a* and 7 *b*).

Activity Studies.—Recombined chains were separated from excess, unre-

combined L chains on sephadex G-100 (above). Aliquots were added to native, unlabeled anti-DNP and standard precipitin curves were performed with the mixtures and DNP-bovine gamma$_2$ globulin (DNP-BγG). The percentage of recombined labeled L chains precipitated at equivalence for each of the specimens tested is listed in Table IV.

As may be seen from the last 2 columns of the table no significant precipitation of the recombined chains occurred in the presence of a heterologous precipitating system. In the presence of anti-DNP–DNP-BγG the γH-Ab$L^{I^{125}}$, $\gamma L^{I^{131}}$ recombinants showed a similarly slight precipitation. The $AbL^{I^{125}}$, $\gamma L^{I^{131}}$ mixture, unrecombined with any H chains also showed negligible precipitation in this assay (2.4 $\pm$ 1 per cent). The AbH-$AbL^{I^{125}}$, $\gamma L^{I^{131}}$ recombinants were, however, active. Significantly, twice as many of the AbH-$AbL^{I^{125}}$ recombinants

TABLE V

Specific Adsorption of Antibody Chain Recombinants on a DNP-BγG-Streptomycin Precipitate

Specimen	Recombined in the presence of hapten		Recombined in the absence of hapten	
	I^{125} adsorbed	I^{131} adsorbed	I^{125} adsorbed	I^{131} adsorbed
	per cent	*per cent*	*per cent*	*per cent*
AbH-$AbL^{I^{125}}$, $\gamma L^{I^{131}}$	57	40	27	12
$AbL^{I^{125}}$, $\gamma L^{I^{131}}$	3	3		

formed in the presence of hapten were precipitated as those formed in the absence of hapten. It is also clear that AbH-$\gamma L^{I^{131}}$ recombinants were active. Again, the favorable influence of hapten during the reassociation process is evident.

Similar results were obtained in the assay employing streptomycin-precipitated antigen. In this assay, however, 20 to 30 per cent of the γH-Ab$L^{I^{125}}$, $\gamma L^{I^{131}}$ recombinants were adsorbed, presumably non-specifically. A similar percentage of the AbH-$AbL^{I^{125}}$, $\gamma L^{I^{131}}$ recombinants remained adsorbed in the presence of hapten (see Materials and Methods). The percentage of recombinants whose precipitation was inhibitable by the DNP-aminocaproate hapten ("specific adsorption") is listed in Table V. The data agree well with those obtained with the previous assay. Again, those recombinants formed in the presence of hapten showed twice the activity found with those recombinants formed in the absence of hapten.

DISCUSSION

The data presented show that the separated chains of gamma$_2$ globulin are able to recombine in an orderly manner. This is demonstrated by the finding

that the recombined chains travel in a single peak with a sedimentation velocity equivalent to that of the native molecule, and that this material contains the correct H:L chain ratio. These findings are in agreement with those of Roholt *et al.* (3) and the recently reported studies by Olins and Edelman (20) and Gally and Edelman (21). These authors showed that the soluble portion of their recombined material had the immunoelectrophoretic mobility (3), sedimentation constant (3, 20, 21), antigenic properties, and papain cleavage products (20, 21) similar to those of the native molecules.

H chains alone tend to form aggregates under the conditions of dialysis used. When H chain monomers (*H'* in Fig. 1) are used for recombination, 90 per cent of the H chains are recoverable as recombined molecules. Using pooled H chains (*H* plus *H'* in Fig. 1) only about 60 to 70 per cent of the H chains are recovered in recombined form. This result might be in part due to residual unseparated L chains in the pooled H chain fraction. However, under the conditions used for chain separation such L chain contamination of the H chains has been found to be less than 5 per cent (15). A more likely explanation is that aggregated H chains can recombine with L chains less readily than can single H chains. This is illustrated by the finding that few L chains were found to have recombined with aggregated H chains sedimenting faster than the 7S peak (see Table I). On the other hand, the pooled L chains are all capable of recombining at least in the presence of excess H chains (Fig. 3).

Once brought to pH 5.0 or above in 0.10 to 0.20 M NaCl the recombined chains are stable. The addition of labeled L chains to recombined unlabeled materials at pH 5.0 allows no exchange of L chains (22). This indicates that the L chains and H chains are firmly combined.

In the presence of excess L chains the total amount of reassociation was constant regardless of the presence or absence of hapten. In the absence of hapten the antibody H chains showed little or no preference for recombining with antibody L chains compared to non-specific L chains. This agrees with previous findings (3) of the non-specificity of the H and L chain interaction (in the absence of specific antigens), a non-specificity apparently sufficient to allow association among chains of different species (20).

In the presence of DNP-aminocaproate, however, the anti-DNP H chains preferentially reassociated with anti-DNP L chains, even though non-specific L chains were present in equivalent amounts (Table II). It has been shown that the dissociation of the H and L chains of anti-DNP can be partially inhibited by the presence of a DNP hapten (8). This suggests that the antibody H chain-antibody L chain interaction can be enhanced by the presence of antigen. The present studies indicate that this enhanced interaction is sufficiently selective to permit the antibody H chains to reassociate preferentially with antibody L chains in the presence of non-specific L chains.

The observations on the activity of the isolated chains and recombinants conducted in this study confirm several previous findings: (*a*) L chains alone or

recombined with non-antibody H chains show no appreciable activity, while antibody H chains retain some measure of activity in the absence of antibody L chains (1–4, 8, 20, 23). (*b*) Antibody H chains recombined with antibody L chains show an enhanced activity over that shown by H chains in the absence of antibody L chains (1–3).

The significant new finding is that the anti-DNP H chains and anti-DNP L chains recombined in the presence of hapten were twice as active as those reassociated in the absence of hapten. Thus, the hapten induced both a change in the chain composition of the recombinants and a more favorable orientation and/or conformation of the recombined chains.

The data in Tables IV and V demonstrate that considerable amounts of AbH-$\gamma L^{I^{131}}$ recombinants were specifically precipitated or adsorbed by antigen. The degree to which this finding can be accounted for by residual antibody H chain activity and/or by hybrid molecules having one antibody L chain and one non-specific L chain, is currently under investigation.

The mechanism of the effect of hapten observed in these studies is not clear. As mentioned above, L chains alone or recombined with non-antibody H chains show little or no antigen-binding capacity. Recombination experiments (1–3), however, show that the antibody L chains can influence activity, and evidence obtained by labeling of antibody-combining sites directly implicates the participation of L chains in the combining sites (24, 25). One mechanism by which hapten could, therefore, enhance the antibody H chain-antibody L chain association would be by way of an interaction between an antigen-combining site on the L chain and a hapten–H chain complex. The interacting of the antibody L chains with the antibody H chain–hapten complex could also serve to orient the L chains more favorably. On removal from dissociating conditions these recombinants would be stable even in the absence of hapten.

Alternatively the effect of hapten could be exclusively on the H chain. Its presence could induce a more favorable (re)folding of that chain, causing its presumed binding site(s) for L chains to reform in a more specific way. The latter sites would presumably have strong L chain *binding* activity but relatively little *specificity* for particular L chains except when the H chain was properly folded.

The present experiments were performed using subunits derived from antibodies. There is nothing in these studies which implies *de novo* antibody-combining site induction by antigen (26). No activity was found in γH-AbL or γH-γL recombinants formed in the presence of hapten.

The possible significance of these findings with respect to *in vivo* antibody synthesis is difficult to assess. The *in vitro* conditions used are clearly artificial. Still, they imply that the antigen–antibody-subunit interaction itself can provide sufficient free energy release for the selective formation of a unique multichain molecule with enhanced activity. It is interesting to speculate that *in vivo* the

antigen by bringing about such subunit associations could lead to the release of the mechanism by which these subunits were formed in the first place, thereby stimulating synthesis of those chains. It is possible, moreover, that enzyme substrates could similarly influence the association of enzyme subunits. It should go without saying that our *in vitro* experiments may be consistent with the first part of this speculaton but of course provide no evidence with respect to the latter points.

SUMMARY

Conditions were developed by which the separated H and L chains of $gamma_2$ globulins recombined to form four-chained molecules in good yields.

In the absence of antigen, anti-2,4-dinitrophenyl (anti-DNP) H chains randomly reassociated with a mixture of antibody and non-specific $gamma_2$ globulin L chains. In the presence of a specific hapten, however, the antibody H chains preferentially interacted with the anti-DNP L chains.

Antibody H chain–antibody L chain recombinants formed in the presence of hapten were more active than the corresponding recombinants formed in the absence of hapten.

Speculations are made regarding the possible mechanisms and biological significance of these effects.

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