PREFERENTIAL RECOMBINATION OF ANTIBODY CHAINS TO FORM EFFECTIVE BINDING SITES*

By O. A. ROHOLT, PH.D., G. RADZIMSKI, AND D. PRESSMAN, PH.D.

(From the Department of Biochemistry Research, Roswell Park Memorial Institute‡, Buffalo, New York)

(Received for publication, May 13, 1965)

The heavy (H) and light (L) polypeptide chains of γG-immunoglobulin[1] recombine to form units similar to the intact globulin molecule (1, 2). When the chains come from antibody, antibody activity is recovered (1, 3, 4). The recombination of H and L chains of globulins has been shown to take place for several animal species; in fact, chains even from different species will combine (5). We have recently reported (6) that there is an individual (or perhaps genetic group) specificity among rabbits in that the combination of H and L chains of antibody molecules gives a much better recovery of hapten-binding activity when both chains are derived from the same serum pool than when the H and the L chains are each from pools of sera involving different rabbits. This specificity in recombination to form active antibody was shown with anti-*p*-azobenzoate and with anti-*p*-azobenzenearsonate antibodies.

We have now carried out experiments involving the chains from the whole γG-immunoglobulin fraction of anti-*p*-azobenzenearsonate serum [IgG_{Fr} (anti-Rp)][1] and studied the antibody activity of the recombined chains. The recovery of antibody activity was higher than would have been expected if random recombination between the H and L chains from the anti-Rp antibodies and the other globulin molecules in this fraction had occurred. This experiment indicated a preferential recombination of the antibody globulin chains so that reformation of antibody units was favored, rather than hybrids involving the chains of anti-Rp antibody and the chains of the other globulin molecules present.

---

* Presented at the Annual Meeting of the American Association of Immunologists, Atlantic City, New Jersey, April 1965. Supported in part by the United States Public Health Service, grant AI 03962 from the National Institute of Allergy and Infectious Diseases.

‡ A unit of the New York State Department of Health.

[1] The following abbreviations are used: The 7S γ-globulin is designated by IgG or γG-immunoglobulin as suggested by the memorandum on "Nomenclature for Human Immunoglobulins" in the *Bull., World Health Organ.* 1964, **30**, 447. The γG-immunoglobulin *fraction* of an antiserum, for example, of anti-Rp serum, is designated as IgG_{Fr} (anti-Rp); of normal rabbit serum as IgG_{Fr} (NRS). The corresponding specifically purified *antibody* is designated as IgG_{Ab} (anti-Rp). The antibody-depleted IgG-fraction of an anti-Rp antiserum is designated as IgG_{Fr} (depleted anti-Rp).

Rp stands for *p*-azophenylarsonate; Xp stands for *p*-azobenzoate. Anti-Rp and anti-Xp refer to the corresponding antibody or serum.

We also carried out experiments concerning the interaction of chains from specifically purified IgG-antibody of individual rabbits with chains from the antibody-depleted γG-immunoglobulins of the same rabbits that provided the antibody as well as with the chains from the γG-immunoglobulin fraction of normal rabbit serum [IgG_{Fr} (NRS)].[1]

It was found that there was a greater strength of combination of the antibody chains when they recombined among themselves than when they combined with the chains from other IgG molecules. This can be called a greater specificity of combination for the chains from antibody.

Materials and Methods

Preparation of Specific Antibody and Antibody-Depleted γG-Immunoglobulin.—Specifically purified rabbit antihapten antibody was prepared from serum of rabbits injected repeatedly with either *p*-azobenzenearsonate (Rp) or *p*-azobenzoate (Xp) bovine γ-globulin conjugate (7).

The IgG_{Fr} (antihapten)[1] or IgG_{Fr} (NRS)[1] was prepared from serum by a sodium-sulfate fractionation similar to the procedure of Kekwick (8) but using successive precipitations at 18, 14, and 12½ per cent sodium sulfate. This procedure yields a product essentially free of γM-immunoglobulins.

Anti-Xp antibody [IgG_{Ab}(anti-Xp)] was removed from the IgG_{Fr}(anti-Xp) by adsorption onto a conjugate of an insoluble polymer of rabbit serum albumin and *p*-azobenzoate (Xp-poly RSA) (9). Two portions of 150 mg adsorbent were used in sequence for 340 mg globulin. The mixture was agitated gently for 1 hour in the cold room for each adsorption. After the second adsorption of the IgG_{Fr}(anti-Xp) by the Xp-poly RSA, the unadsorbed, antibody-depleted IgG_{Fr}[1] was dialyzed against several changes of borate buffer (10), centrifuged, and filtered. The anti-Xp antibody was isolated from the two batches of antibody-loaded Xp-poly RSA prepared above. They were mixed and washed repeatedly with borate buffer: saline (1:9). The final wash was essentially free of protein (the absorbancy at 280 mμ was 0.014). The adsorbent was then washed once with saline and the adsorbed antibody was eluted with 25 ml of ice cold 0.16 M glycine buffer of pH 2.65. The suspension was stirred occasionally for 10 minutes in order to desorb the antibody. In order to decrease the amount of nonspecific protein entrained, the pH of the suspension was brought to 8.1 by the addition of 1.7 ml of 3 M tris buffer of pH 8.6 (72.6 gm tris and 21 ml concentrated HCl in 200 ml solution, pH adjusted with concentrated HCl). Thus, the antibody was readsorbed in the presence of less non-specific protein than in the initial adsorption. The suspension was agitated gently in the cold for 1 hour and then centrifuged. The absorbancy at 280 mμ of the supernate was 0.077. The adsorbent was washed five times with borate buffer:saline (1:9) and once with saline. The absorbancy of the final wash was 0.018. The adsorbent was suspended in 30 ml of the pH 2.65 glycine buffer in an ice bath and centrifuged. The supernate was removed and the adsorbent treated twice more with 15 ml of the glycine buffer. The pooled supernates (63 ml) were brought to pH 8 with 4 ml of 3 M tris buffer of pH 8.6 and allowed to come to room temperature. The eluted protein was precipitated by adding 13.4 gm of solid Na_2SO_4. The suspension was centrifuged and the precipitate taken up in borate buffer and dialyzed against several changes of borate buffer.

The IgG_{Ab} (anti-Rp) and the IgG_{Fr}(depleted anti-Rp) were prepared similarly using the conjugate of rabbit serum albumin polymer and *p*-azobenzenearsonate (9). Anti-Rp antibody was also prepared from its specific precipitate with Rp-ovalbumin conjugate by dissolving the precipitate in benzenearsonate solution and separating the antibody from the Rp-ovalbumin on a column of diethylaminoethylcellulose (11).

Preparation of H and L Polypeptide Chains of Rabbit γG-Immunoglobulin.—H and L polypeptide chains were obtained by reduction and alkylation according to the method of Fleischman, Pain, and Porter (12) using 0.2 M mercaptoethanol and 10 per cent excess of iodoacetamide. Separation of the H and L chains was done on sephadex G-100 equilibrated with 1 M propionic acid. In general this gives two H chain peaks which we designate as H_1 and H_2 (6).

Determination of Hapten-Binding Activity by Equilibrium Dialysis.—Equilibrium dialysis was carried out as previously described (13) using I^{125}-labeled *p*-iodobenzenearsonate and *p*-iodobenzoate for anti-Rp and anti-Xp antibodies, respectively.

Radioimmunoelectrophoresis.—This was performed as described by Onoue, Yagi, and Pressman (14) using goat antiserum against rabbit H and L chains.

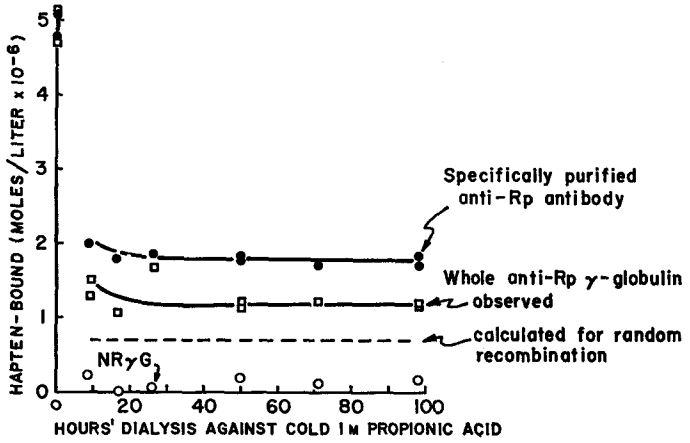

FIG. 1. Hapten-binding activity of reduced and alkylated anti-*p*-azobenzenearsonate antibody after exposure to propionic acid solution. The points at zero hours were not exposed to propionic acid.

RESULTS

Effect of Reduction, Alkylation, and Period of Exposure to Propionic Acid on the Hapten-Binding Activity of IgG_{Ab}(Anti-Rp) and IgG_{F_T}(Anti-Rp).—

IgG_{Ab} (anti-Rp) and IgG_{F_T} (anti-Rp) were reduced, alkylated, and dialyzed against 1 liter cold borate buffer overnight and then against two successive 4 liter portions of cold saline in 24 hours. A relatively small amount of precipitate formed and was centrifuged down. The concentration of the supernate was adjusted to 1 mg/ml for the anti-Rp antibody and 7 mg/ml for the IgG_{F_T} (anti-Rp). Portions (0.4 to 0.5 ml) of each preparation were placed in individual dialysis bags. Two bags containing each preparation were placed in 400 ml of cold 1 M propionic acid and the rest of the bags in cold saline. At set times, one or two bags containing each preparation were transferred to 400 ml of 1 M propionic acid and those bags exposed longer than 24 hours were transferred to a fresh 400 ml portion of 1 M propionic acid after 24 hours. Ninety-eight hours after the first bags had been placed in the propionic acid solution, all of the bags were transferred from the acid solution to a 4 liter volume of cold borate buffer which was changed once. Thus, the periods of exposure of the reduced and alkylated preparations to the cold propionic acid solution were from 0 to 98 hours. After the

second equilibration with the borate buffer, hapten-binding measurements were made on the contents of the bags by equilibrium dialysis (13).

The results are shown in Fig. 1. Reduction and alkylation of anti-Rp antibody or of IgG_{Fr} (anti-Rp) without exposure to the acid, has no effect on the hapten binding (1). However, exposure to 1 M propionic acid for even 10 hours (the time the bag was in the propionic acid bath) resulted in a drop of hapten-binding activity for both preparations which did not change further during 90 more hours of exposure. It is important, however, that the binding activity

TABLE I

Recovery of Hapten-Binding Activity of IgG_{Ab} (Antihapten) Alone and with Respective IgG_{Fr} (Depleted Antihapten) after Reduction, Alkylation, and Treatment with Propionic Acid

Antibody	Relative hapten-binding activity*			
	IgG_{Ab} alone	IgG_{Fr}‡ alone	$IgG_{Ab} + IgG_{Fr}$‡	
			Observed	Calculated
Anti-Rp				
No. 3524	60	1	62	9§
No. 3527	53	2	38	8§
Anti-Xp				
Pool	53	1	44	26‖

* Untreated antibody = 100 in each case; concentration of IgG_{Ab} (antihapten), 1 mg per ml in each case. Free *p*-iodobenzenearsonate concentration = 10.7×10^{-6} M, free *p*-iodobenzoate concentration = 18.3×10^{-6} M.

‡ IgG_{Fr} (depleted antihapten), (see footnote 1).

§ $IgGA_b$:IgG_{Fr} = 1:6.

‖ IgG_{Ab}:IgG_{Fr} = 1:1.

of IgG_{Fr}(anti-Rp) leveled off at 25 per cent of the activity of the original although about 6 times as much L and H chain is present from the nonspecific globulin as from the antibody. This is not much less than the value of 36 per cent for the recovery of activity of the anti-Rp antibody which did not contain normal globulin. If random recombination between hapten-specific L and H chains and the chains from non-specific globulin occurred, less than one seventh of the activity would be recovered since we previously found that such hybrid combinations show very low hapten-binding activity. This is appreciably lower than the value of 25 per cent observed here and suggests that completely random recombinations did not occur, but rather there was preferential recombination between hapten-specific L and H chains.

In another experiment, portions of IgG_{Ab} (anti-Rp) and IgG_{Ab} (anti-Xp) were reduced and alkylated. Other portions were individually mixed with the respective IgG_{Fr} (depleted anti-Rp or Xp). The mixtures were reduced, alkylated, and exposed to cold 1 M propionic acid as described in the previous experiment. After 36 hours each solution was dialyzed against water, saline, and finally borate buffer. Hapten-binding measurements were made on each preparation by equilibrium dialysis.

The results are shown in Table I. The last two columns compare the observed recovery of activity with the recovery calculated for random combination between H and L chains from antibody and from the depleted globulin from the same serum.

TABLE II

Hapten-Binding Activity of Treated IgG-Fraction of Anti-p-Azobenzenearsonate Serum

Preparation	Relative binding activity*
IgG_{Fr} (anti-Rp), intact	100
Reduced, alkylated IgG_{Fr} (anti-Rp)	
Not treated with propionic acid	101
Treated with propionic acid	
Chains not separated	34
Chains separated	
H_1(Rp) + L(Rp)	27
H_2(Rp) + L(Rp)	28

* Binding measured at free *p*-iodobenzenearsonate concentration of 15.1×10^{-6} M.

The recovery of hapten-binding activity in the case of each of the mixtures of antibody with the antibody-depleted globulin is greater than that calculated for random combination. In fact, in one case the presence of the depleted globulin did not decrease the observed binding by antibody chains alone at all and showed little effect in the others. Here again there is a preferred combination of antibody H and L chains.

However, two other possibilities exist to explain the observed retention of binding activity, (*a*) dissociation of the H and L chains was not complete in the propionic acid solution, or (*b*) hapten-specific L (or H) chains combine with nonspecific H (or L) chains but when the nonspecific chains are from the same animal, the combination gives good binding activity contrary to the results observed when they come from different rabbits (1).

Experiments were carried out to check these possibilities.

Hapten-Binding Activity of H and L Chains of IgG_{Fr}(Anti-Rp) after Separation and Recombination.—

H_1 and H_2 chains prepared from IgG_{Fr} (anti-Rp) were individually mixed with L chains from the same source on an equimolar basis. The mixtures were made in the same ratio of

relative molar absorbance at 280 mμ as found for the total H chain to L chain yield obtained off the sephadex G-100. The mixtures were then dialyzed against cold water and borate buffer and hapten-binding measurements made.

The results are shown in Table II. The recovery of activity upon mixing the separated chains is in close agreement with that observed when the chains were not separated; *i.e.*, 27 to 28 per cent compared to 34 per cent. This shows that

TABLE III

Relative Binding Activity of Various Mixtures of H and L Chains from IgG_{Ab} (Anti-Xp), IgG_{Fr} (Depleted Anti-Xp) and IgG(NRS)*

H chain	L chain		
	Anti-Xp antibody	IgG_{Fr} (depleted anti-Xp)	IgG(NRS)
Anti-Xp antibody			
H_1	82	8	7
H_2	80	7	7
IgG_{Fr} (depleted anti-Xp)			
H_1	3	4	2
H_2	3	4	1
IgG(NRS)			
H_2	2	2	1
None	1	1	0

* Untreated anti-Xp antibody = 100; untreated IgG_{Fr} (depleted anti-Xp) = 3. The protein concentration during the equilibrium dialysis was 1 mg per ml and the free *p*-iodobenzoate concentration = 18.8×10^{-6} M.

the chains apparently achieve the same dissociation equilibrium in propionic acid regardless of whether they were separated and mixed or not. Again it appears that specific antihapten H and L chains preferentially combine with one another even in the presence of non-specific counterpart chains. It is interesting that the same L chain preparation combines effectively, on an equimolar basis, with both fractions of H chains (H_1 or H_2).

Hapten Binding of H(Xp) and L(Xp) Chains Mixed with Non-Specific H and L Chains from the Same Serum.—The possibility that hapten-specific H (or L) chains and non-specific L (or H) chains from the same rabbit combine to give good binding activity was tested. It was shown to be *not* the case in the following experiment involving anti-Xp instead of anti-Rp antibody.

H(Xp) and L(Xp) chains from serum from a single rabbit were mixed with non-specific H and L chains prepared from the IgG-fraction of the same serum

after removal of the anti-Xp antibody or mixed with the chains from non-immunized rabbits.

Equimolar amounts of H and L chains in 0.02 M propionic acid were mixed in an ice bath and 0.05 M propionic acid added to give a total protein concentration of 1 mg per ml. The solutions were stored in the refrigerator for 18 hours and then 0.4 to 0.5 ml portions of each solution, in duplicate, were placed in dialysis bags. These were dialyzed against cold 0.05 M propionic acid for 5 hours and then against 4 liters of borate buffer overnight. The bags were transferred to a borate buffer solution of I^{125}-labeled *p*-iodobenzoate for measurement of the hapten-binding activity of each of the various solutions by the method of equilibrium dialysis.

TABLE IV

Effect of L(N) Chains on the Hapten-Binding Activity of H(Xp) + L(Xp) Chains

Preparation	Equivalents of each chain*			Hapten bound‡	Relative binding activity§
	H_2(XP)*	L(Xp)	L(N)		
a	1	1	0	7.27‖	0.79
b	1	5	0	7.17	0.78
c	1	1	1	5.82	0.63
d	1	1	2	5.04	0.55
e	1	1	3	4.50‖	0.49
f	1	1	6	3.46	0.38
g	1	1	9	3.25‖	0.35
h	1	0	9	1.12‖	—
i	0	1	0	0.39	—
j	0	1.5	0	0.35	—
k	0	5	0	1.25	—
l	0	0	3	0.26	—
m	0	0	9	0.50	—

* H chain equivalent to 1.33 mg antibody per ml; based on absorbancy at 280 mμ and with the ratio of molar extinction coefficients for H to L chains taken as 3:1.

‡ Moles per liter $\times 10^6$ with free hapten concentration = 18.0×10^{-6} M.

§ Relative to Xp-antibody (hapten bound = 9.17×10^{-6} M).

‖ Duplicate determinations with a deviation from the mean of less than 3 per cent.

The results are shown in Table III. The combination of hapten-specific H and L chains resulted in more than 80 per cent recovery of the binding activity relative to the untreated antibody. The hapten-binding activity of the specific H chains combined with L chains from either the Xp-antibody–depleted globulin of the same rabbit or with L chains from the globulin of different unimmunized rabbits both showed essentially the same low binding activity; *i.e.*, 7 to 8 per cent of the untreated antibody. The values of 3 to 4 per cent for combinations involving H chains from the antibody-depleted globulin and L chains from either the antibody or from the same globulin were so low as to indicate

only low levels of contamination by antibody remaining in the depleted globulin. The value of 3 per cent for the binding by the untreated antibody-depleted globulin also suggests little, if any, such contamination.

Thus, these results show that for H and L chain combinations, good binding activity requires that both the L and H chains be derived from specific antibody and that combination with L and H chains from non-specific globulin does not yield effective sites even when from the same serum (5).

Competition between L(Xp) and L(N) Chains for H(Xp) Chains.—The effectiveness of L chains from IgG(NRS)[1] in competing for H(Xp) chains with L(Xp) chains originally separated from these particular H(Xp) chains was investigated.

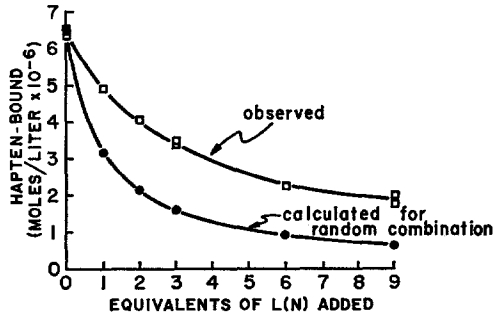

FIG. 2. Effect of L(N) chains on the recombination of L(Xp) and H_2(Xp) chains. One equivalent each of H_2(Xp) and L(Xp) chains are present throughout.

Several individual volumes of 0.05 M propionic acid each containing L(Xp) chains equivalent to 0.67 mg of intact Xp antibody were mixed with different volumes of an L(N) chain solution so that the molar ratios for L(N) to L(Xp) chains ranged from 0:1 to 9:1. To each mixture was added a volume of 0.05 M propionic acid containing H_2(Xp) chains equivalent to 0.67 mg of intact Xp antibody. The L(Xp) and H_2(Xp) were from the same antibody preparation while the L(N) chains were from a pool of serum from rabbits other than these. The volume of each solution was brought to 0.5 ml with 0.5 M propionic acid and the solutions allowed to stand in the refrigerator for 40 hours and then each solution was dialyzed against cold borate buffer. Hapten-binding measurements were carried out on each solution by equilibrium dialysis. Portions of each sample were analyzed by radioimmunoelectrophoresis (14)

The results of the binding measurements by equilibrium dialysis shown in Table IV (preparations *a* through *g*) show a reduction in the hapten-binding activity in the presence of excess L(N) chains. However, as shown in Fig. 2, this reduction is much less than would be expected if L(N) chains competed equally with L(Xp) chains and resulted in inactive combinations. The bottom curve in Fig. 2 is the theoretical binding activity based on the above assumptions and plotted against the added L(N) chains. The upper curve shows the observed binding which is appreciably greater. Thus there is either preferential

combination of H_2(Xp) and L(Xp) or the H_2(Xp)-L(N) combination binds at a great enough level to give the observed binding. The binding of hapten by this latter combination is shown by preparation *h* (Table IV) where 1 mole of H_2(Xp) in the presence of 9 moles of L(N) binds 1.12×10^{-6} moles of hapten. The addition of only 1 mole of L(Xp) (preparation *g*) increases the binding to 3.25×10^{-6}, almost half the value for the H_2(Xp)-L(Xp) combination in the absence of L(N) (preparation *a*). This shows conclusively a preferential combination of H_2(Xp) and L(Xp).

The control binding data on Table IV show that L(Xp) chains alone (prep-

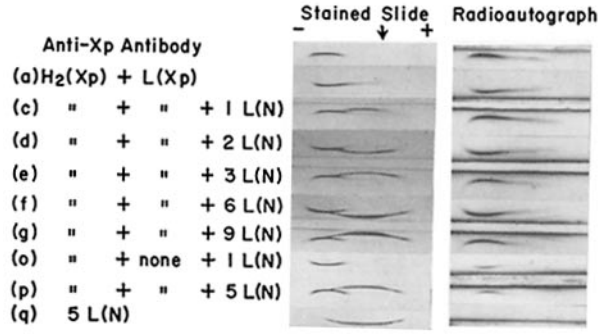

FIG. 3. Radioimmunoelectrophoretic patterns of recombinants in various propionic acid–treated mixtures of H_2(Xp) chains, L(Xp) chains, and L(N) chains. The letters in parentheses refer to the preparations on Table IV. Patterns *o*, *p*, and *q* are representative of preparations *h* to *m* on Table IV. The arrow (↓) shows the position of the starting well. Approximately 2 μl of solution containing 1 mg of antibody per ml or an equimolar amount of H_2(Xp) chains (along with the indicated amounts of L chains) were applied in each case.

arations *i*, *j*, *k*) and L(N) chains alone (preparations *l*, *m*) all have low hapten-binding activity. Excess L(Xp) chains do not increase the hapten-binding activity relative to that for an equivalent amount of L(Xp) chains (compare preparations *a* and *b*).

The immunoelectrophoresis patterns (Fig. 3) show that the amount of combined H and L material did not vary significantly whether there were equivalent amounts of H_2(Xp) and L(Xp) chains or even a 9-fold excess of L(N) chain. The excess L chains migrated in a typical manner in each case and appeared as an additional arc.

On extension to radioimmunoelectrophoresis (Fig. 3), the radioautographs show binding by the combination H(Xp) + L(Xp). Although additional L(N) does not appear to decrease the degree of binding, we would expect a decrease by a factor of only 2 on the basis of the binding data on Table IV and this is too small to be detected by this technique as currently applied.

DISCUSSION

When the H and L chains from specifically purified anti-*p*-azobenzenearsonate (Rp) antibody were recombined, 36 per cent of the hapten-binding activity of the untreated antibody was recovered. When the separated chains from the whole γG-immunoglobulin fraction of the anti-Rp serum were combined, almost as much (27 per cent) of the activity of the untreated Rp-glob-

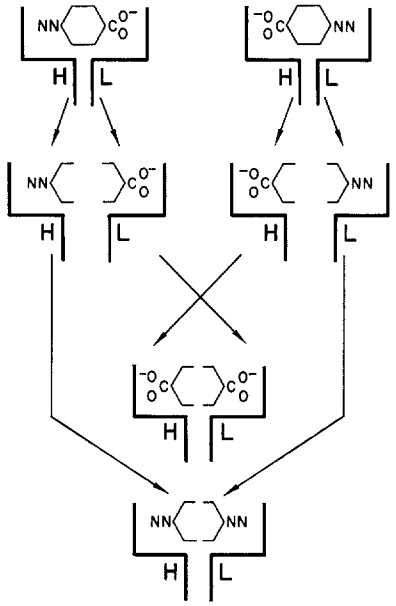

FIG. 4. Hypothetical cross-combination of H and L chains from two different anti-*p*-azobenzoate antibodies to yield antibodies of hybrid specificity not effective for *p*-azobenzoate.

ulin was recovered in spite of the presence of a relatively large concentration of chains from other globulin molecules not specific for this hapten.

In the case of other preparations of specific antibody, the recovery of antigen-binding activity by the recombined chains was even higher (Table II) and the presence of chains from non-specific globulin molecules again had little effect on the recovery of binding activity. The combination of the chains from hapten-specific antibody to give active antibody is apparently but little effected by the presence of these non-specific chains.

The fact that the recovery of binding activity by the antibody chain mixtures was less than 100 per cent of that of the respective untreated specifically purified antibody preparations may actually be because some of the recombinants involved H and L chains which initially came from antibody molecules directed against different parts of the haptenic group and therefore did not

form effective sites.[2] The decrease in activity was not appreciably more than this when non-specific γG-immunoglobulin was present in much higher proportion and even greater opportunity would seem to exist for ineffective H and L combinations to take place. However, since there is preferred combination between those H and L chains which give effective anti-Rp or anti-Xp sites, we would expect the same situation to exist for H and L chains which give effective antibody against other determinants. The globulin other than the specific antibody concerned here probably contains antibodies against other substances and for each of these, we would expect the chains to recombine with each other in a preferential manner also. Even normal globulins would probably exist in groups, for which there would be preferential combination of H and L chains derived therefrom. Thus, the high concentration of H and L chains not from hapten-specific antibody would not be completely free to combine randomly, *i.e.*, compete with hapten-specific H and L chain, but would actually tend to remove themselves from this competition though preferential combination among their own various types. The per cent of hapten-binding activity recovered would then reflect the over-all degree to which preferential combination occurred between the H and L chains derived from within each type of globulin present.

The recovery of hapten-binding activity, and presumably the degree to which preferential combination takes place between the H and L chains from specifically purified antibodies, varies from antibody preparation to preparation. Thus, in the experiment referred to in Fig. 1, H and L chains of the anti-Rp antibody showed only 36 per cent of the activity of the original antibody on recombination while in another experiment, the recombination of H and L chains from anti-Xp antibody led to a recovery of 79 per cent (preparation *a*, Table IV). The anti-Xp antibody recombinants in Table III showed a recovery of hapten-binding activity of 80 per cent and in Table I, showed recoveries

[2] The combination of H and L chains to form a binding site approximating the original site seems to require either a correct alignment of the two chains to form the binding site or the modulation of one chain by the other to form a binding site (4, 15). It is not necessary that the two chains which combine to yield a site come from the same molecule, since in our recombination experiments we have found that effective sites are formed upon mixing H chain and L chain preparations made from different portions of the same pool of serum. In this case the H and L chains could not have come from the same molecule.

It seems possible that two chains from very closely related antibody molecules might cross-combine to form a site closely related to the original, either by cooperative site formation or by modulation. However, antibodies formed by a single rabbit, even against a single hapten, are known to be heterogenous in specificity (16) and it is not readily apparent how the chains from any two molecules involved in this type of heterogeneity could recombine in an exchange manner to form an active site. For example, as shown schematically in Fig. 4, if for one anti-Xp antibody site the L chain were directed against the azo group and in another site the L chain were directed against the carboxylate group, cross-combination would not yield an effective site.

between 50 and 60 per cent. In other experiments, not reported in detail here, in which the chains from antibody from individual rabbits were combined, we found recoveries of activity ranging from 30 to 90 per cent of the activity of the untreated original antibody. The higher recoveries of activity may be because all of the H and L chains are relatively homogenous with respect to their ability to recombine effectively or else the chains from each of several types of antibody present combine well within groups so that relatively few ineffective cross-combinations occur.

Non-specific L chains can compete with specific L chains for specific H chains. The presence of non-specific L chains in an equimolar mixture of H and L chains from a preparation of Xp-antibody, resulted in an appreciable decrease in the hapten-binding activity of the latter (Table IV). In this case, there was an excess of L chains over H chains and there was successful competition. If a stoichiometric amount of the non-specific H chains were present, it would have preferentially combined with the non-specific L chains and removed them from the competition.

Slight enhancement of the antibody activity of H chains by the addition of non-specific L chains has been reported in the case of antibodies directed against other determinants (4, 17). Franek *et al.* (17) reported that for antibodies from cattle, L chains from non-specific γ-globulin can produce the same "activation" of the antibody H chains as do L chains from the antibody, if the non-specific L chains are added in excess. Their report concerned the system, bovine anti-dinitrophenyl (anti-DNP) antibody and bovine γ-globulin chains.

In our work, mixtures of L(N) chains with H(Xp) chains, using either one equivalent (Table III) or even nine equivalents (Table IV, preparation *h*) of L(N) chains resulted in only about 10 per cent of the hapten-binding activity of that attained when one equivalent of L(Xp) chains were mixed with the H(Xp) chains and it is questionable whether the activity of such hybrids is actually any greater than that of H chain preparation alone. For this system it is certainly low at best.

When we added excess hapten-specific L chains to the H(Xp) chains (Table IV, preparation *b*) there was little effect on the hapten-binding activity compared to that with one equivalent (preparation *a*).

The difference between our results and those of Franek *et al.* (17) may be due to differences in the animal species or to the behavior of the chains from antibodies directed against the DNP-determinant, or both.

Metzger and Mannik (18) found that in the presence of hapten, H chains from rabbit anti-DNP antibody combined with anti-DNP L chains rather than with non-specific L chains. In the absence of hapten, the combination was random. The DNP-haptenic group is not ionic and is specifically bound to the H chain of anti-DNP antibody (19) at the low pH values where chains start to combine appreciably with each other. This is in contrast to the negatively

charged haptenic groups used in our work which lose their charge at low pH values and are not bound to specific antibody under the conditions where chains are coming together. Our antibody preparations are hapten-free and preferred combination does take place.

These workers also reported (20) a random combination of H and L chains from anti-DNP antibody from rabbits of different allotypes. These rabbits, although of different allotypes, may nevertheless be isotypic with respect to a genetic group which permits effective combination of the L and H antibody chains from allotypically different rabbits. Their results may also reflect a property of the anti-DNP antibody.

SUMMARY

The recovery of hapten-binding activity by a mixture of H and L polypeptide chains of the whole γG-immunoglobulin fraction from rabbit anti-*p*-azobenzenearsonate (Rp) serum is almost as great as that by a mixture of H and L chains from specifically purified Rp antibody. Random combination among the H and L chains from the anti-Rp antibodies and the normal γG-immunoglobulin present would result in little recovery of hapten-binding activity. This suggests a preferential recombination of H and L chains from antibody. Mixtures of H or L chains from anti-*p*-azobenzoate (Xp) antibody and the complementary chains from antibody-depleted γG-immunoglobulin show little hapten-binding. When anti-Xp antibody H chains are added to mixtures of one equivalent of anti-Xp L chain and increasing amounts of non-specific L chain, the hapten-binding by the mixtures decreases, but not as much as if the H chains combined with the L chains randomly. Hapten was not present during these recombination procedures. These data indicate that in the cases of anti-Xp and of anti-Rp antibodies, there is a selective combination between those H and L chains which give effective hapten binding regions.

The technical assistance of Mrs. L. Emerson, Mr. J. Gurreri, and Mr. D. Park is gratefully acknowledged.

BIBLIOGRAPHY

1. Roholt, O., Onoue, K., and Pressman, D., Specific combination of H and L chains of rabbit γ-globulins, *Proc. Nat. Acad. Sc.*, 1964, **51**, 173.
2. Olins, D. E., and Edelman, G. M., Reconstitution of 7S molecules from L and H polypeptide chains of antibodies and γ-globulins, *J. Exp. Med.*, 1964, **119**, 789.
3. Franěk, F., and Nezlin, R. S., Recovery of antibody by interaction of different peptide chains isolated from purified horse antitoxin, *Folia Microbiol. Prague*, 1963, **8**, 128.
4. Edelman, G. M., Olins, D. E., Gally, J. A., and Zinder, N. D., Reconstitution of immunologic activity by interaction of polypeptide chains of antibodies, *Proc. Nat. Acad. Sc.*, 1963, **50**, 753.

5. Fougereau, M., Olins, D. E., and Edelman, G. M., Reconstitution of antiphage antibodies from L and H polypeptide chains and the formation of interspecies molecular hybrids, *J. Exp. Med.*, 1964, **120,** 349.
6. Roholt, O., Radzimski, G., and Pressman, D., Polypeptide chains of antibody: effective binding sites require specificity in combination, *Science*, 1965, **147,** 613.
7. Nisonoff, A., and Pressman, D., Heterogeneity and average combining constants of antibodies from individual rabbits, *J. Immunol.*, 1957, **80,** 417.
8. Kekwick, R. A., The serum proteins in multiple myelomatosis, *Biochem. J.*, 1940, **34,** 1248.
9. Onoue, K., Yagi, Y., and Pressman, D., Immunoadsorbants with high capacity, *Immunochemistry*, 1965, **2,** 181.
10. Pressman, D., Brown, D. H., and Pauling, L., The serological properties of simple substances. IV. Hapten inhibition of precipitation of antibodies and polyhaptenic simple substances, *J. Am. Chem. Soc.*, 1942, **64,** 3015.
11. Koshland, M. E., Englberger, F., and Koshland, Jr., D. E., A general method for the labeling of the active site of antibodies and enzymes, *Proc. Nat. Acad. Sc.*, 1959, **45,** 1470.
12. Fleischman, J. B., Pain, R. H., and Porter, R. R., Reduction of γ-globulins, *Arch. Biochem. and Biophysics*, 1962, suppl. 1, 174.
13. Grossberg, A. L., and Pressman, D., Nature of the combining site of antibody against a hapten bearing a positive charge, *J. Am. Chem. Soc.*, 1960, **82,** 5478.
14. Onoue, K., Yagi, Y., and Pressman, D., Multiplicity of antibody proteins in rabbit anti-*p*-azobenzenearsonate sera, *J. Immunol.*, 1964, **92,** 173.
15. Roholt, O., Grossberg, A., Onoue, K., and Pressman, D., Relative contributions of H and L chains to the binding site of antibody molecules, presented at the 6th International Congress of Biochemistry, International Union of Biochemistry, New York, 1964, abstract No. II-159.
16. Kreiter, V. P. and Pressman, D., Fractionation of anti-*p*-benzenearsonate antibody by means of immunoadsorbents, *Immunochemistry*, 1964, **1,** 91.
17. Franěk, F., Kotynek, O., Sĭmek, L., and Zikán, J., S-sulfonated anti-dinitrophenyl antibodies. Some specific features of the interaction between isolated H and L subunits, International Symposium on the Cellular and Molecular Basis of Antibody Formation, Prague, 1964.
18. Metzger, H., and Mannik, M., Recombination of antibody polypeptide chains in the presence of antigens, *J. Exp. Med.*, 1964, **120,** 765.
19. Metzger, H., and Singer, J. S., Binding capacity of reductively fragmented antibodies to the 2,4-dinitrophenyl group, *Science*, 1963, **142,** 674.
20. Mannik, M., and Metzger, H., Hybrid antibody molecules with allotypically different L-polypeptide chains, *Science*, 1965, **148,** 383.