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Isolation of Purified H and L Polypeptide Chains from Guinea-Pig γ_2-Immunoglobulin after Mild Reduction

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Summary. A method is described whereby biologically active H and L chains, free from each other by immunochemical criteria, can be prepared from guinea-pig γ_2-immunoglobulin. The procedure makes use of mild reduction and alkylation in the absence of a denaturing agent, followed by gel filtration in the presence of 4 M guanidine·HCl.

Specific binding of antigen by the method of equilibrium dialysis was found only when specific H chains were mixed with L chains, with more activity resulting when the L chains were also specific. Specific binding by antibody H chains alone was not found.

INTRODUCTION

Ever since the initial experiments of Edelman and Poulik (1961) showing that the γG-immunoglobulin (IgG) molecule is composed of more than one kind of polypeptide chain, there has been considerable interest in developing methods for isolating them. Two general approaches have been developed: extensive reduction and fractionation in the presence of a denaturing agent (Edelman and Poulik, 1961), and mild reduction, in which principally interchain disulphide bonds are cleaved, followed by fractionation in acid (Fleischman, Pain and Porter, 1962) or detergent (Utsumi and Karush, 1964). The first approach was successfully exploited by Small, Kehn and Lamm (1963) who used 5 M guanidine·HCl as the denaturing agent. This method enables recovery of pure heavy (H) and light (L) chains which are suitable for physicochemical studies, but which are insoluble in the usual aqueous media and hence are unsuitable for studies of biological properties. When the second approach is employed, the H and L chains obtained maintain their biological properties, but a variable degree of contamination of the H chains with L chains has been noticed (Fleischman, Porter and Press, 1963; Utsumi and Karush, 1964; Nussenzweig, Franklin and Benacerraf, 1964; Nelson, Noelken, Buckley, Tanford and Hill, 1965).

The present report describes a procedure, designed to yield H and L chains with a high degree of purity and also intact biological properties, which combines features of the two approaches used previously. Guinea-pig γ_2-immunoglobulin is mildly reduced according to Fleischman *et al.* (1962), but separation is carried out on a Sephadex G-200 column in 4 M guanidine·HCl. A similar method was used by Franěk, Kotýnek, Šimek and Zikán (1965), who reduced IgG with sulphite and used acid and urea for filtration. Their products were not characterized as to purity.

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MATERIALS AND METHODS

Gamma$_2$-immunoglobulin was obtained by exposing guinea-pig γ-globulins (Pentex) batchwise to DEAE-cellulose in the presence of 0·01 M phosphate buffer, pH 8·0 (Nussenzweig and Benacerraf, 1964). The supernate, which was shown by immunoelectrophoresis to contain only γ_2-immunoglobulin, was dialysed against 0·001 M phosphate buffer, pH 7·6, and lyophilized. Anti-dinitrophenyl (DNP) antibodies were obtained from the sera of guinea-pigs immunized with DNP-bovine γ-globulin (Benacerraf, Ovary, Bloch and Franklin, 1963) by precipitation at equivalence with DNP-bovine fibrinogen in the presence of 0·01 M Na_2-ethylenediaminetetraacetate, followed by elution of the washed precipitate with 0·1 M 2,4-dinitrophenol (DNPOH) in the presence of streptomycin. In order to prepare pure antibody, the soluble antibody–DNPOH complex was passed through a column of Dowex-1 (X8; 200–400 mesh) equilibrated with 0·15 M NaCl–0·01 M phosphate, pH 7·4, which removes free and dissociable hapten (Farah, Kern and Eisen, 1960).

A column of Sephadex G-200 equilibrated with 4 M guanidine·HCl was prepared as follows. An approximately 7 M solution of guanidine·HCl (Eastman Organic Chemicals, 'highest purity') was repeatedly exposed to decolorizing carbon until the OD/cm at 280 mμ was less than 0·07. Sephadex G-200 was thoroughly fined in water to remove small particles and then allowed to equilibrate overnight with approximately 4 M guanidine·HCl before packing the $50 \times 2{\cdot}8$ cm column. Guanidine solutions of exact molarity were made according to refractive index measurements, using the data of Kielley and Harrington (1960).

Immunoglobulins were reduced essentially as described by Fleischman *et al.* (1962). Two per cent protein solutions in 0·5 M tri-sacetate buffer, pH 8·2, were made 0·05–0·1 M in dithioerythritol (DTE) (Cleland, 1964), and reduction was allowed to proceed for 1 hour at room temperature, following which alkylation was achieved by adding a two-fold excess of iodoacetamide (recrystallized from petroleum ether and ethanol). The reduced and alkylated protein was dialysed against saline to remove any remaining reducing agent, and the small amount of precipitate which formed during dialysis was discarded. The protein solution was then dialysed against 4 M guanidine·HCl, which contained about 0·001 M iodoacetamide (to alkylate any free sulphhydryl groups exposed when the protein was unfolded in the guanidine). The sample was made 4 per cent in sucrose and then layered over the Sephadex. Filtration was carried out at room temperature. About 7-ml samples were collected at timed intervals, and the eluate was monitored at 280 mμ. In some instances, protein fractions were filtered through the column a second time. Appropriate samples were pooled, concentrated by ultrafiltration and dialysed against saline. Guinea-pig H chains were perfectly soluble in saline, but appreciable amounts of L chains precipitated upon removal of the guanidine unless the solution of L chains was kept dilute. If the concentration was below 1 mg/ml, less than 10 per cent of the L chains were lost by precipitation. The greater solubility of the H chains is characteristic of guinea-pig γG-immunoglobulin and does not reflect the use of guanidine since fractionation of reduced and alkylated guinea-pig immunoglobulins by the method of Fleischman *et al.* (1962) yields products with similar solubility properties and since fractionation of reduced and alkylated rabbit γG-immunoglobulin in guanidine yields H chains which are, as expected, less soluble than L chains (Wilheim and Lamm, 1965). On one occasion protein from the H chain peak was extensively reduced in 0·3 M β-mercaptoethanol in the presence of 6 M guanidine·HCl (Small *et al.*, 1963).

In one experiment protein from the rear two-thirds of the L chain peak after one passage through the Sephadex column was labelled with ^{131}I (Thorbecke, Maurer and Benacerraf, 1960), and 0·5 mg of this was added to 53 mg of intact γ_2-immunoglobulin just before reduction. In a different experiment another 0·5 mg of this labelled protein was by itself exposed to the reducing and alkylating agents and then filtered through the column. ^{131}I was counted in a well scintillation counter.

Specific precipitation by double diffusion was performed in 0·5 per cent agarose made up in 0·05 M phosphate buffer, pH 7·6 (Ouchterlony, 1953), using antisera prepared in rabbits against whole guinea-pig γ_2-globulin, its F(ab')$_2$ fragment produced by pepsin digestion (Nisonoff, Wissler, Lipman and Woernley, 1960), or against guinea-pig H and L chains.

Specific precipitation in liquid media was carried out in the antibody excess zone using ^{131}I-labelled guinea-pig γ_2-globulin and its purified H chain together with antisera specific for guinea-pig L chains, type λ*, or whole guinea-pig γ_2-globulin. An aliquot of ^{131}I-labelled antigen was brought to 0·2 ml volume with saline, and 0·3 ml of the antiserum added. The mixture was left at 37° for 30 minutes and then stored at 4° for 72 hours. The precipitate was washed three times with cold saline, and then centrifuged at 3000 rev/min for 30 minutes. The supernatant and all washings were saved for counting. The precipitate was dissolved in 0·1 N NaOH. Aliquots of the supernatant and the dissolved precipitate were counted.

Sedimentation velocity was determined in the Spinco model E ultracentrifuge at 59,780 rev/min and 19°.

Equilibrium dialysis was performed at room temperature according to the general description of Eisen (1964) with [^{3}H]DNP coupled to ε-aminocaproic acid (DNP-EACA) as the hapten. The DNP-EACA had a specific activity of 19·5 mc/mM, and was prepared by Dr W. Paul. Initial volumes of 0·8 ml were used, both inside and outside the dialysis bag. From $2{\cdot}6$–30×10^{-10} moles of hapten were employed, resulting in about 6000 count/min/10^{-9} moles of hapten when 0·45 ml of hapten solution was added to 15 ml of phosphor and counted in a Packard Tri-Carb liquid scintillation counter. Amounts of protein ranged from 84 to 600 μg. Mixtures of H and L chains were made in molar ratios of 1:1 or 1:2. Protein concentrations were determined from optical density readings using an $E^{1\%}_{280} = 13{\cdot}3$, and the moles of antibody combining sites were taken to be equal to the moles of H chain, assuming a molecular weight of 5×10^4. When only L chains were employed, for comparison purposes moles of protein were calculated assuming a molecular weight of 2×10^4. After equilibrium had been reached, the concentration of hapten both inside and outside the bag was measured. The change in volume during dialysis was determined by weighing the dialysis bag and its contents before and after the dialysis. About 2 per cent of the hapten was bound to the dialysis bag in these experiments.

RESULTS

After reduction, alkylation, and a single gel filtration, the elution patterns were similar for both non-specific immunoglobulins and for anti-DNP antibodies which had been freed of remaining traces of hapten by passage through Dowex. Recoveries of protein eluted

* Guinea-pig L chains consist of two families (κ and λ) containing distinct antigenic determinants. Approximately one-fourth of non-specific guinea-pig γ_2-immunoglobulin molecules contain the λ type (Nussenzweig, Lamm and Benacerraf, 1965).

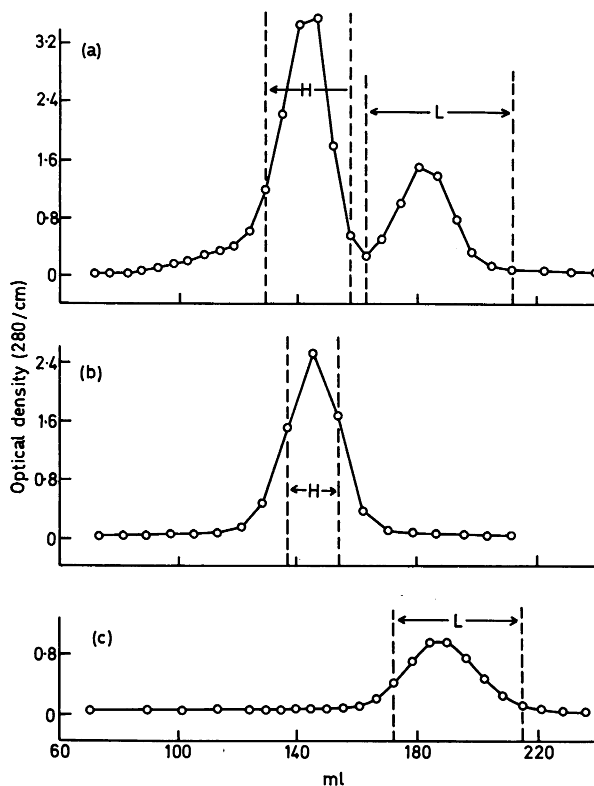

Fig. 1. (a) Elution pattern of non-specific guinea-pig γ_2-immunoglobulin from the Sephadex G-200 column run in 4 M guanidine after mild reduction and alkylation.

(b) A second filtration of the indicated portion of the H chain peak of panel A. The indicated tubes contained H chains that were pure by immunochemical criteria. About 1 per cent of the optical density was eluted, where L chains would be expected. This is not evident in the figure because of the reduction in scale.

(c) A second filtration of the indicated portion of the L chain peak of panel A. The indicated tubes contained pure L chains. About 1·5 per cent of the optical density was eluted where H chains would be expected. Again, this is not evident because of the reduction in scale.

from the column averaged 68 ± 7 per cent of the material originally reduced. This percentage recovery takes into account losses during reduction, alkylation, dialysis against saline, dialysis against guanidine, addition of sucrose, and gel filtration. A typical elution pattern is given in Fig. 1(a). There is slight assymmetry of the leading edge of the H chain peak (probably indicative of dimers, non-specific aggregates, or unreduced protein). The L chain peak comprised $29{\cdot}5\pm0{\cdot}5$ per cent of the total protein eluted (assuming equal extinction coefficients for the H and L chains).

When protein from the leading edge and peak tube of the H chain fraction after mild reduction was re-reduced extensively in the presence of 6 M guanidine·HCl and re-filtered, less than 1 per cent of the protein was eluted where L chains would be expected. Nevertheless, when the peak tubes from the H chain peak and the latter half of the L chain peak, after partial reduction, were separately pooled, dialysed against saline, concentrated, and tested by double diffusion in agarose, lack of purity was indicated (Fig. 2): H chains gave a reaction of identity with intact IgG, similar to previous results obtained in this laboratory

(Nussenzweig and Benacerraf, 1964; Nussenzweig *et al.*, 1964) with both guinea-pig and human IgG reduced and fractionated by the method of Fleischman *et al.* (1962), and some of the H chains showed contamination with L chains. In addition, if enough protein was placed in the L chain well, contamination with H chain determinants could also be demonstrated.

Thus, contamination of the H and L chain fractions after a single filtration was clearly demonstrable by the highly sensitive Ouchterlony technique although the extent of contamination probably did not exceed 2 per cent by chromatographic criteria (see below for the results of second filtrations). Considerable purification was therefore achieved by this initial filtration in the 4 M guanidine, which decreased chain interactions. Complete resolution of the H and L chains was still not achieved, however, and required a second filtration.

When protein from the rear two-thirds of the L chain peak after a single passage through Sephadex was labelled with ^{131}I and then passed through the column with newly-reduced γ_2-immunoglobulin, about 15 per cent of the radioactivity came out before the L chain peak. When the peak tubes from the H chain fraction of this effluent (about 20 mg of protein) were again passed through the column, the radioactivity was about equally divided between the H and L peaks, although protein as monitored by optical density at 280 mμ was detected only in the H chain peak because of the small amount of labelled L chains used. These experiments were thought to indicate that the rear two-thirds of the L

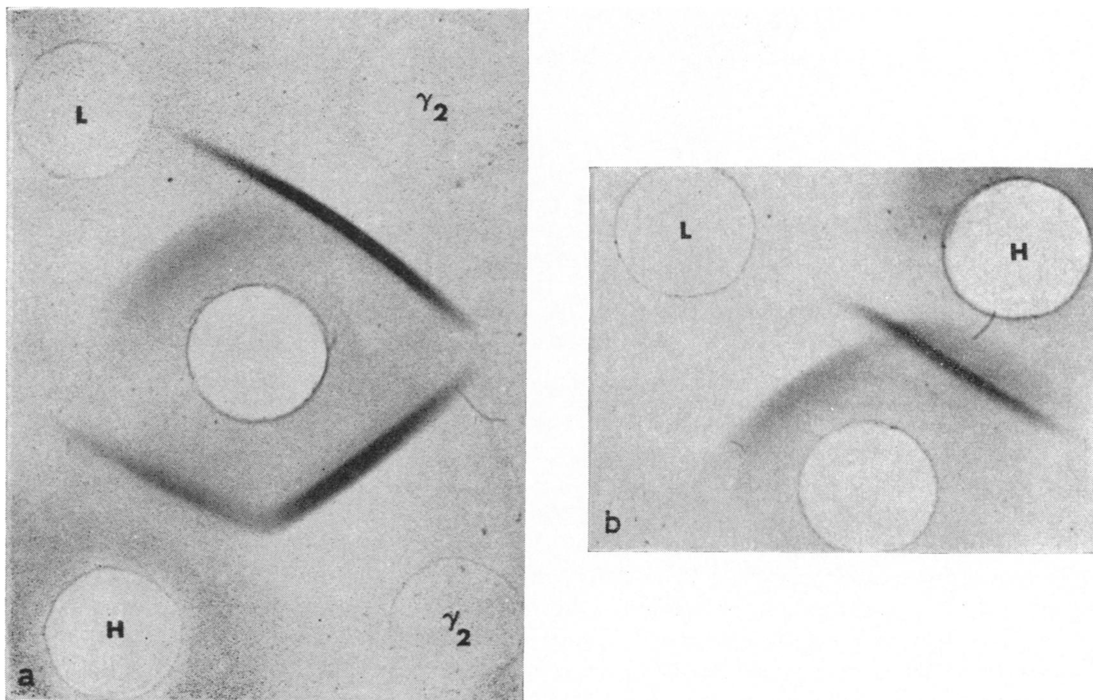

FIG. 2. Specific precipitation in agarose gel. H and L: the respective polypeptide chains obtained after a single passage of reduced, alkylated non-specific guinea-pig γ_2-globulin through the column; γ_2: non-specific guinea-pig γ_2-globulin. In (a) the central well contains rabbit anti-F(ab')$_2$ of guinea-pig γ_2-globulin. In (b) the antibody well contains a rabbit antiserum against guinea-pig H and L chains.

chain peak after a single filtration of mildly reduced γ_2-immunoglobulin were about 7–8 per cent contaminated with H chains. In support of this reasoning, when the radioactive protein was filtered by itself, 7 per cent of the radioactivity was eluted where H chains would be expected. However, the actual degree of contamination may well have been less than 7 per cent because of artifacts related to the iodination procedure such as aggregation of some of the L chains.

Because a single passage of reduced and alkylated γ_2-immunoglobulin through the Sephadex column was not sufficient for preparing pure H and L chains, even in the presence of denaturing agent, the peak tubes from the H chain peak and the entire L chain peak after a single filtration were separately concentrated and then refiltered (Fig. 1b and c). In this instance each peak eluted was symmetrical. In addition to the symmetrical main peaks shown in Fig. 1(b and c), there were small amounts of protein (between 1 and 2 per cent) where L chains would be expected in Fig. 1(b) and where H chains would be expected in Fig. 1(c). These impurities, which are not readily apparent in the figure because of the reduction in scale, probably account for the results of the gel diffusion studies shown in Fig. 2.

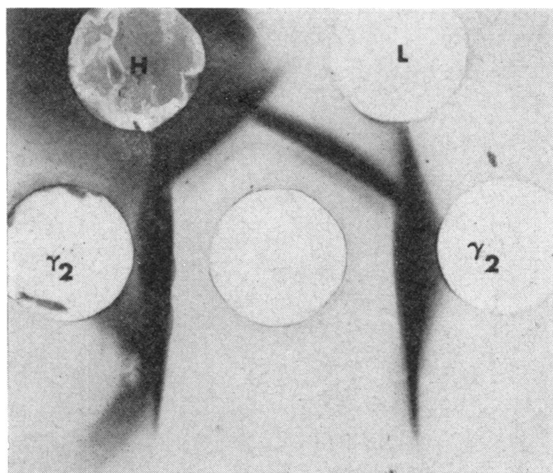

Fig. 3. Specific precipitation in agarose gel. Centre well: rabbit anti-F(ab′)$_2$ of guinea-pig γ_2-globulin. γ_2: guinea-pig γ_2-globulin. H and L: the respective purified chains from non-specific guinea-pig γ_2-globulin after two passages through the column. Identical patterns were obtained with a rabbit anti-guinea-pig γ_2-globulin antiserum.

After concentration of the final H and L chain fractions and dialysis against saline, gel diffusion studies gave evidence of purity (Fig. 3). The H chains gave a reaction of non-identity with L chains and both H and L chains gave a reaction of partial identity with intact γ_2-immunoglobulin. Additional evidence of H chain purity was obtained by specific precipitation reactions using ^{131}I-labelled antigens (Table 1). Rabbit antiserum against the λ type of guinea-pig L chain precipitated even less H chain than did the normal rabbit serum used as a control. This same antiserum precipitated the expected amount of non-specific guinea-pig γ_2-immunoglobulin, which contains about 25 per cent of molecules with the λ type of L chain (Nussenzweig *et al.*, 1965). In similar previous experiments (Nussenzweig *et al.*, 1964) up to 50–60 per cent of human H chains prepared

TABLE 1

SPECIFIC PRECIPITATION OF GUINEA-PIG γ_2-IMMUNOGLOBULIN AND ITS ISOLATED H CHAINS, LABELLED WITH ^{131}I, BY RABBIT ANTISERA IN THE ZONE OF ANTIBODY EXCESS

Antigen	(μg)	Anti-type λ L chain			Anti-γ_2-globulin			Normal rabbit serum		
		Supernate (counts/min)	Precipitate (counts/min)	Percentage precipitated	Supernate (counts/min)	Precipitate (counts/min)	Percentage precipitate	Supernate (counts/min)	Precipitate (counts/min)	Percentage precipitated
H_{γ_2}*	80	259,230	15,810	5·7	29,850	233,460	89	281,420	18,860	6·3
	160	1,330,000	22,375	1·7				1,265,000	68,925	5·2
γ_2	50				1,888	28,119	94			
	75	97,393	29,089	23				123,318	1,175	0·94
	150	199,409	58,408	23						
	225	282,100	88,383	24						

* The reactions with H_{γ_2} were performed on separate occasions allowing for decay of the label.

by the method of Fleischman *et al.* (1962) were precipitated by combined antisera against κ and λ Bence Jones proteins.

When analysed by sedimentation velocity at a concentration of 1·7 mg/ml, the purified L chains migrated as a single symmetrical peak with a sedimentation coefficient of 3·9S, indicating that dimers were the principal species. Purified H chains (5·3 mg/ml) also sedimented as a single peak, but this was diffuse and assymmetric, indicative of marked heterogeneity. Most of the material had sedimentation coefficients in the neighbourhood of 7·3S. Both preparations had previously been frozen for a month.

By these methods H and L chains with a similar degree of purity could also be prepared from guinea-pig anti-DNP antibodies freed of hapten by the technique of Farah *et al.* (1960).*

After demonstration that the method described in this paper enables the preparation of purified H and L chains that retain their properties as antigens, it was of interest to study them as antibodies. Ability to combine with tritiated DNP-EACA was investigated by means of equilibrium dialysis. The results of these experiments are listed in Table 2. The original preparation of anti-DNP antibodies had a binding constant of about 10^7 litres/mole. When specific H chains were tested by themselves for binding activity, the sites occupied were always less than 1 per cent and showed no consistent relationship to the ratio of total hapten to total sites. The small amount of binding to specific H chains alone is therefore probably due to experimental errors and/or non-specific binding. The specific L chains by themselves likewise exhibited no binding activity. However, when specific H and specific L chains were mixed in a saline medium, hapten binding was demonstrated corresponding to occupancy of up to about 5 per cent of the potential sites, with association constants in the range of 10^5. When specific H and specific L chains were mixed in saline, dialysed against 4 M guanidine·HCl, and then brought back slowly to saline by successive dialysis against decreasing concentrations of guanidine (in an effort to allow a more efficient recombination), hapten binding was increased to an occupancy of as much as 9 per cent of the potential sites, again with association constants of about 10^5. For a given ratio of hapten to potential sites, the latter type of experiment (slow re-association during removal of the guanidine) resulted in increased binding of hapten. Binding of hapten to specific H and non-specific L chains mixed in saline was at best minimal, and the low degree of apparent binding may be largely non-specific or result from experimental errors. The binding activity of specific H and non-specific L chains after slow removal of guanidine is apparently specific since the percentage of sites occupied was always greater than 1 and increased with increasing ratios of hapten to sites. A mixture of L chains from anti-DNP antibodies and non-specific H chains was devoid of binding activity.

DISCUSSION

Mild reduction of guinea-pig γ_2-immunoglobulin followed by a single gel filtration in the presence of 4 M guanidine·HCl resulted in two peaks containing about 70 per cent and 30 per cent of the eluted protein. The leading edge of the first peak was slightly asymmetric, indicating the probable presence of residual native IgG or aggregated protein. In

* When anti-DNP antibodies with bound hapten still present were mildly reduced, alkylated and filtered once in 4 M guanidine·HCl, only 17 per cent of the eluted protein was in the L chain peak. Such gross contamination of the H chain peak with L chains under these circumstances is in agreement with the findings of Metzger and Singer (1963), who used the method of Fleischman *et al.* (1962).

TABLE 2

EQUILIBRIUM DIALYSIS STUDIES, USING [^{3}H]DNP-EACA, ON ANTI-DNP GUINEA-PIG ANTIBODIES, ISOLATED SPECIFIC AND NON-SPECIFIC H AND L CHAINS, AND COMBINATIONS OF THESE CHAINS

Protein	Total moles hapten / Moles total sites*	Percentage total sites* occupied by hapten
Intact anti-DNP γ_2	0·32	28·0
	1·1	65·0
H_{DNP}	0·033	0·13
	0·10	0·00
	0·16	0·75
	0·35	0·00
	0·39	0·64
	1·8	0·24
L_{DNP}	0·06	0·06
	0·21	0·00
$H_{DNP}+L_{DNP}$†	0·034	2·2
	0·10	1·6
	0·16	2·8
	0·35	2·3
	0·39	5·3
	1·8	5·2
$H_{DNP}+L_{DNP}$‡	0·10	5·0
	0·35	8·1
	2·4	9·0
$H_{DNP}+L_{\gamma 2}$†	0·10	0·53
	0·35	0·86
$H_{DNP}+L_{\gamma 2}$‡	0·11	1·1
	0·37	1·3
	1·8	3·9
$H_{\gamma 2}+L_{DNP}$	0·11	0·24
	0·36	0·00
$H_{\gamma 2}+L_{\gamma 2}$	0·11	0·00
	0·36	0·24
$H_{\gamma 2}$	0·11	0·07
	0·36	0·00
$L_{\gamma 2}$	0·050	0·00
	0·17	0·00

* The number of sites was determined from the number of moles of H chain present, using 50,000 as the molecular weight and assuming that each H chain contained a single site. When only L chains were present, the number of 'sites' for purposes of comparison was taken to be the number of L chains present, using a molecular weight of 20,000.

† The isolated chains were mixed in saline and allowed to stand for a few hours before equilibrium dialysis.

‡ The isolated chains were first mixed in saline, and then made 4 M in guanidine·HCl, which was subsequently slowly dialysed out.

addition, the main portions of the H and L chain fractions were shown not to be pure; however, this must have resulted from insufficient resolution during the gel filtration rather than from too tight binding of H and L chains since second, separate filtrations of the L chain fraction and of the peak tubes from the H chain fraction led to products that were pure by immunological criteria. In gel diffusion studies by the Ouchterlony method, the H and L chain precipitation lines gave reactions of non-identity with each other and reactions of partial identity with native γ_2-immunoglobulin. In addition, H chains were not precipitated by anti-L chain antiserum in a liquid medium.

Binding studies of H and L chains after reduction and fractionation of specific antibodies have led to conflicting results (see the recent review of Cohen and Porter, 1964). Most workers agree that L chains, by themselves or in the presence of non-specific H chains, have no activity. The questions at large are whether H chains alone are responsible for the binding of antigen, whether and how much the activity of H chains can be enhanced by the presence of specific or non-specific L chains, and whether both specific H chains and either specific or non-specific L chains are needed. Attempts to investigate these problems have undoubtedly been hindered by the difficulty in obtaining H chains free of L chains. The high degree of purity of the H and L chains obtained by the method described in this study allowed this problem to be re-examined. The small amounts of binding indicated in Table 2 for specific H chains alone and for specific H chains mixed in saline with non-specific L chains are for reasons discussed above, interpreted largely to be the result of experimental errors and/or non-specific binding. Although unable to bind antigen, purified H chains could be 89 per cent precipitated by antiserum against whole γ_2-immunoglobulin (Table 1).

In this study significant binding of antigen was found only when specific H chains were present with L chains. In this regard, specific L chains were more effective than non-specific L chains. After recombination of specific H and specific L chains a maximum of about 9 per cent of the potential binding sites were occupied by hapten. If this binding activity is compared to that of native antibody at a similar ratio of hapten to sites (Table 2), the recovered activity may be considered a maximum of about 29 per cent (8·1/28). Compared to the results of other similar studies, our findings agree most closely with those of Franěk *et al.* (1965), whose H chains by themselves also showed no binding activity for the DNP determinant.

These data lend support to the view that both L chains and a portion of the H chain, the Fd-fragment, contribute to the antibody specificity of IgG immunoglobulins.

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