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The Binding of Haptens by the Polypeptide Chains of Rabbit Antibody Molecules

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1. The binding of haptens by the polypeptide chains derived from two rabbit immunoglobulin G antibodies was examined by gel chromatography and equilibrium dialysis. 2. The γ chains were examined in a dilute sodium acetate buffer, pH5.4, in which they exist as a monodisperse solution of dimers; aggregation of the protein promoted by some haptens had to be avoided. These chains exhibited variable extents of binding, reflecting the specificities of the parent antibody molecules, usually with only small increments above the binding by γ chains from normal immunoglobulin G. 3. The light chains existed as an interconverting mixture of monomers and dimers in all buffers of near neutral pH that were examined. They bound small amounts of hapten, again broadly reflecting the specificities of the parent antibody molecules. 4. For both the γ and light chains the dimeric state appeared necessary for appreciable binding of hapten. Apparently in each case the partners in the dimer interact in a manner analogous to the γ chain–light chain interaction in the parent antibody molecule, to give a site analogous to the antibody site. This implies that the binding of antigens by isolated chains has a large fortuitous element, providing no reliable indication of their contributions to the original antibody sites.

The nomenclature used in this paper is that recommended by the World Health Organization (1964). 'Normal' IgG* refers to that obtained from unimmunized rabbits.

Amino acid sequences of immunoglobulin molecules (Milstein & Pink, 1970) reveal analogous variable regions in the heavy and light chains, suggesting that both chains are involved in making up the site for the remarkably variable function of antibody activity. Affinity labelling of antibody sites has also tended to implicate both chains, although in any single experiment residues from one or another chain might acquire most of the label (Thorpe & Singer, 1969). It is anomalous then that although there are many reports that isolated γ chains (the heavy chains of IgG) can bind appreciable amounts of homologous antigen (Jaton *et al.*, 1968; Painter *et al.*, 1972; earlier work reviewed by Fleischman, 1966; Cohen & Milstein, 1967), only a few workers (Goodman & Donch, 1965; Mangalo & Raynaud, 1967; Yoo *et al.*, 1967; Painter *et al.*, 1972) have succeeded in demonstrating such activity among the light chains. Sometimes the extents of binding by isolated chains have been used to assess the energetic contributions which each chain makes to binding by the parent molecules (Utsumi & Karush, 1964; Painter *et al.*, 1972); such studies have again suggested a predominant role for the heavy chains.

* Abbreviations: IgG, immunoglobulin G; anti-Dnp, antibody to the 2,4-dinitrophenyl determinant; anti-ABS, antibody to the 4-azobenzene-1-sulphonate determinant.

This paper describes the specific binding of haptens by the γ and light chains from two antibody populations raised in rabbits. The low or negligible extents of binding by light chains reported by previous workers were found to be partly explicable by the binding being confined to chain dimers in an interconverting system of monomers and dimers. In examining the binding by γ chains attention has been paid to preventing contamination by whole-antibody molecules, and to keeping the chains monodisperse. Experiments with Fd fragments suggested that binding by the whole γ chains is also dependent upon dimerization, confirming the conclusion reached by Painter *et al.* (1972).

It is concluded that despite some quantitative differences binding by heavy and binding by light chains are basically similar phenomena, unlikely to give any useful quantitative information about chain contributions to intact antibody sites.

Materials and Methods

Dithiothreitol (A grade) was obtained from Calbiochem, Los Angeles, Calif., U.S.A. Iodoacetamide was recrystallized from ethanol. Sodium deoxycholate (Laboratory Reagent; British Drug Houses Ltd., Poole, Dorset, U.K.) and ϵ-Dnp-lysine ('chromatographically pure') from Mann Research Laboratories, New York 6, N.Y., U.S.A., were used as supplied. 2,4-Dinitrophenol (British Drug Houses Ltd.) was recrystallized from ethanol. Methyl

Orange (4'-dimethylaminoazobenzene-4-sulphonate; British Drug Houses Ltd.) was recrystallized as the sodium salt from hot water.

4-(*N*-Chloroacetyl-L-tyrosine-azo)benzene-1-sulphonate was prepared by allowing *N*-chloroacetyl-L-tyrosine (Mann Research Laboratories) to react at 0°C and pH 10 with the diazo derivative of sulphanilic acid, stoicheiometric quantities being used. After 60min the pH was lowered to 7.0 by addition of HCl and the reaction mixture was passed at room temperature through Sephadex G-10 equilibrated with water, the hapten emerging after the buffer salts. The absorption spectrum showed a maximum at 327nm, and the millimolar extinction coefficients were as shown in Table 1.

Rabbit normal IgG was prepared as described by Stevenson & Dorrington (1970).

Anti-Dnp and anti-ABS were raised in rabbits of indeterminate breed. Antigens for immunization were prepared by coupling 2,4-dinitrophenyl or 4-azobenzene-1-sulphonate groups to bovine γ-globulin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) by standard techniques (Little & Eisen, 1967; Nisonoff, 1967). Primary immunization consisted of 5mg in complete Freund adjuvant, divided into four lots injected subcutaneously into each limb. After 6 weeks a booster of 1mg adsorbed to aluminium hydroxide was given intravenously. The animals were bled 1, 2 and 3 weeks later. Anti-Dnp was prepared by the method given by Eisen *et al.* (1967) for antibodies of high affinity. Purified anti-ABS was prepared by adsorption to Sepharose 4B coupled by the cyanogen bromide method (Porath *et al.*, 1967) to 4-(*p*-aminophenylazo)benzenesulphonate (Technical Grade; Eastman Organic Chemicals, Rochester, N.Y., U.S.A.), with subsequent elution by 0.03M-4-(*N*-chloroacetyl-L-tyrosine-azo)benzene-1-sulphonate or 0.01M-Methyl Orange in 0.2M-Tris–HCl, pH8.0, and removal of the bulk of the hapten by passage through Sephadex G-25 equilibrated with the same buffer. Residual hapten was removed promptly when the protein was exposed to propionic acid during the preparation of γ and light chains. Both purified antibodies thus prepared consisted entirely of 7S IgG, as judged by analytical centrifugation and immunoelectrophoresis.

γ and light chains were prepared from normal or antibody IgG by sequential reduction, exposure to 1M-propionic acid and chromatography on Sephadex G-100 as described by Stevenson & Dorrington (1970). It is important that the protein while in propionic acid be at a concentration less than 2mg/ml to encourage complete disruption of the γ chain–light chain non-covalent bonds. The criterion for complete disruption was a yield of light chains, as measured by E_{280} in the propionic acid, equal to 27% of the total recovered material. Such yields were obtained regularly with the anti-ABS, but with the anti-Dnp the yield averaged 24%, indicating that about 11% of the IgG remained unsplit. As is described in the Results section, unsplit IgG had sometimes to be removed from the γ chain but not from the light-chain preparations.

After their separation in 1M-propionic acid the chains were dialysed at 4°C into the buffers used for binding experiments: 4mM-sodium acetate buffer, pH5.4, for the γ chains; for the light chains 0.1M-NaCl with either 4mM-sodium acetate, pH5.4, or 20mM-Tris–HCl, pH8.0. The choice of buffer is critical for the γ chains (for preventing aggregation) but not for the light chains.

Dimers of light chains in which the dimeric state is stabilized by an interchain S–S bond were prepared as described by Stevenson & Dorrington (1970) for human light chains, based on the method of Gally & Edelman (1964). An amount of 100mg of light chains from anti-ABS yielded 59mg, which persisted in the dimeric state in 1M-propionic acid, the criterion for the existence of an interchain S–S bond; this is an appreciably lower yield than given by human light chains. The disulphide-stabilized dimers were transferred by dialysis from 1M-propionic acid into 0.1M-NaCl–0.004-M sodium acetate, pH5.4, with accompanying partial precipitation. The final yield was 41mg.

The Fab and Fc fragments of rabbit IgG were prepared by the method of Porter (1959). Fd' fragments were prepared by combining and modifying methods described by Nisonoff *et al.* (1960) and Fleischman *et al.* (1963). The IgG, 5mg/ml in 0.05M-NaCl–0.07M-sodium acetate buffer, pH4.7, was incubated with pepsin (crystalline, from pig stomach; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) at 37°C for 18h. The solution was chilled and cold 1M-Tris added to pH8.0. It was then applied to a column of Sephadex G-100 (Pharmacia), equilibrated with 0.2M-Tris–HCl buffer, pH8.0. IgG (undigested) and Fab'_2 fragment emerged from the column respectively as a small leading and large second peak, poorly resolved. The Fab'_2 fraction, with obvious contamination by IgG, was concentrated to 2mg/ml, allowed to react with 0.01M-dithiothreitol for 30min at room temperature, and then with 0.022M-iodoacetamide for 10min at room temperature. The mixture was next passed through Sephadex G-100 equilibrated with 0.01M-Tris–HCl buffer, pH8.0: Fab' fragment, now in the reduced monomeric form, separated clearly from IgG. The Fab' fragment was concentrated to 2mg/ml, dialysed against 1M-propionic acid at 4°C, and finally passed at 4°C through Sephadex G-100 equilibrated with 1M-propionic acid. The Fd' fragment, the first major peak emerging, was transferred by dialysis at 4°C into the 4mM-sodium acetate buffer.

Sheep antisera to rabbit Fab, Fc and light chains were raised by using the same schedule and antigen

doses as given above for immunizing rabbits. The anti-Fab serum contained no anti-Fc activity detectable by the Ouchterlony technique. The anti-Fc required absorption with Fab fragment at 0.8mg/ml. An anti-Fd serum was prepared from the anti-Fab by absorption at equivalence with light chains (0.43mg/ml). IgG from the antisera was obtained as for rabbit IgG (Stevenson & Dorrington, 1970). Immunoadsorbents were prepared by coupling the IgG to Sepharose 4B by the cyanogen bromide method, 0.2M-sodium citrate buffer, pH6.5, being used for the coupling stage (Cuatrecasas, 1970). Binding by immunoadsorbent was established by incubating with antigen solution for 24h at 4°C with constant stirring.

Proteins were concentrated where necessary by ultrafiltration through a UM-10 membrane (Amicon, High Wycombe, Bucks., U.K.) or through 8/32in cellophan tubing (Visking).

Ultracentrifugal analyses were performed with a Spinco model E centrifuge equipped with automatic temperature control. Molecular weights were determined by the meniscus-depletion method (Yphantis, 1964; Montgomery *et al.*, 1969), with interference optics. The partial specific volume of the light chains was taken as 0.72ml/g.

Levels of hapten binding were established either by equilibrium dialysis or by chromatography on Sephadex columns. Both procedures were carried out at 4°C. Equilibrium dialysis employed bags of 8/32in Visking cellophan tubing. The tubing was cleaned by simmering in a dilute solution of disodium EDTA for 10min and rinsing with water. Bags containing duplicate or triplicate 4ml lots of protein solution at 0.4–1.0mg/ml (γ chains) or 2.0–5.0mg/ml (light chains) were immersed for 48h in a protein-free external solution that contained the required concentration of free hapten and was of a volume some 50 times that of the combined internal solutions. The external solution was stirred with a magnetic 'flea'. Chromatography employed columns of Sephadex G-100 or G-25 (fine grade), equilibrated with buffer solution containing free hapten at the required concentration. Details of column and sample sizes are given in the Results section. Protein introduced into a column of sufficient size migrates in succession: (1) to an environment of free hapten at the equilibrating concentration, which will be altered by any binding to the protein; (2) to an environment where the equilibrating concentration of free hapten is restored. The emerging protein peak then contains protein, bound hapten and free hapten at the equilibrating concentration. To demonstrate that the ratio of column size to sample size has been sufficient, the eluent immediately before and after the protein peak is shown to contain equal concentrations of hapten, at the equilibrating concentration. The use of Sephadex G-100 in this method offers the additional advantage that different extents of binding associated with protein aggregates of different sizes can be assessed.

After binding was established by dialysis or passage through Sephadex the extinctions of the protein–hapten mixture, and of the protein-free hapten solution with which it was in equilibrium, were measured at 280nm and at a higher wavelength where the hapten

Table 1. *Millimolar extinction coefficients of proteins and haptens*

	E at indicated wavelengths			
Wavelength (nm) ...	280	327	360	465
IgG (mol.wt. 150000)	201	1	0.5	0.3
γ chains (dimers, mol.wt. 104000)	146	0.7	0.4	0.2
Light chains (dimers, mol.wt. 46000)	55	0.4	0.2	0.1
Fd′ fragments* (dimers, mol.wt. 52000)	73			0.1
Fc fragments* (dimers, mol.wt. 52000)				
ϵ-Dnp-lysine	5.7		17.6	
2,4-Dinitrophenol	4.2		13.3	
Methyl Orange	9.2			25.9
4-(*N*-Chloroacetyl-L-tyrosine-azo)-benzene-1-sulphonate	5.6	25.0		

* In the absence of accurate information Fd′ and Fc fragments were assumed each to be exactly half the size of a γ chain, and each to account for half the extinction given by the γ chain.

has an absorption maximum (see Table 1). From these readings, and the extinction coefficients and molecular weights in Table 1, the molar binding values were calculated. No allowance was made for any change in absorbance of the hapten associated with its being bound.

The average association constant for antibody–hapten interaction is defined as the apparent association constant when half the antibody sites are occupied (Karush & Karush, 1971), and so is in fact equal to the reciprocal of free hapten concentration at this point. This concentration was determined graphically for three preparations of anti-ABS by: (1) mixing antibody IgG, 1–2mg/ml in 4mM-sodium acetate buffer, pH5.4, with Methyl Orange equimolar with the antibody sites; (2) putting 4ml portions at 4°C through columns (1.8cm×20cm) of Sephadex G-25 (fine) equilibrated with a series of concentrations of Methyl Orange in the sodium acetate buffer (1μM, 100nM, 10nM, 1nM); (3) determining the molar binding of hapten by antibody emerging from the columns.

Results

Purity and dispersity of the γ chains and their fragments

To detect contaminating IgG, portions of γ chain solutions were concentrated to 20mg/ml and allowed to react in an Ouchterlony plate with a sheep anti-Fab serum which had previously been absorbed at equivalence (0.23mg/ml) with γ chains. (This antiserum proved more useful than a simple antiserum to light chains.) The method could detect IgG at 2% of the total protein. γ chain solutions from normal and anti-ABS IgG never contained detectable IgG but those from anti-Dnp IgG sometimes did. This might be due to stabilization of the γ chain–light chain union by a small amount of tightly bound hapten, ϵ-Dnp-lysine or dinitrophenol, to which the antibody was exposed during preparation (Metzger & Singer, 1963). All detectable IgG was removed from these solutions by immunoadsorption with Sepharose 4B coupled to sheep IgG from the absorbed anti-Fab serum described above.

Solutions of Fd and Fc fragments were freed of contaminating Fab fragment by using the same immunoadsorbent.

γ chains stored at 4°C in 4mM-sodium acetate buffer, pH5.4, form a stable monodisperse solution of dimers (Stevenson & Dorrington, 1970). However, in the presence of haptens ultracentrifugal examination sometimes revealed aggregation of the protein (Fig. 1). This was dependent upon the concentrations of hapten and protein, and the time of contact. The effect was not observed with ϵ-Dnp-lysine, but was noted with Methyl Orange and to a greater extent with some other haptens, for example bis-dinitrophenyl-L-lysine and 9-hydroxy-10-(*p*-azobenzenesulphonate)phenanthrene. It appeared to correlate with a large apolar area on the hapten and with a tendency for the hapten itself to form micelles as judged by deviations from Beer's law (Burkhard *et al.*, 1953). Because of the complication of non-specific binding by aggregated protein, experiments were designed to measure binding only in aggregate-free systems; thus at hapten concentrations above 10μM binding was established by chromatography on Sephadex G-25 in a time too short for aggregation to become significant.

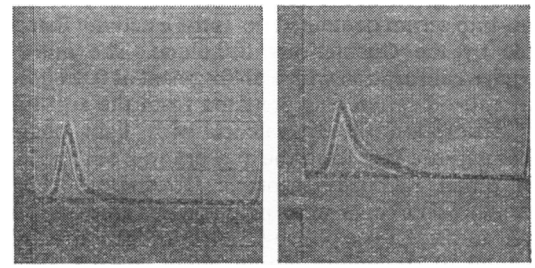

Fig.1. *Aggregation of γ chains in the presence of relatively high concentrations of Methyl Orange*

γ chains from normal IgG, at a final concentration of 6mg/ml, were made 100μM in Methyl Orange in 4mM-sodium acetate buffer, pH5.4. After the stated times of incubation free Methyl Orange was removed from portions (2ml) by passage through Sephadex G-25 (fine) columns (1.5cm×15cm) equilibrated with buffer alone. The middle 1ml of the effluent protein zone, with a protein concentration of about 5mg/ml, was taken for immediate ultracentrifugal examination. All operations were at 4°C. (*a*) After incubation for 15min. (*b*) After incubation for 24h. In each case sedimentation is from the left and conditions were: speed 59780rev./min; temperature 4°C; photograph 35min after reaching full speed; phase plate angle 70°. Values of $s_{20,w}$: (*a*) 4.3S; (*b*) 4.4S. Ultracentrifugal examinations in the presence of 100μM-Methyl Orange gave similar results but the patterns did not photograph well.

Fragment Fd from anti-ABS at 2.2mg/ml in the sodium acetate buffer gave in the ultracentrifuge a single symmetrical peak with $s_{20,w} = 3.55$S, indicative of dimers.

Binding of haptens by the γ chains

Table 2 shows the binding by γ chains from normal and antibody IgG preparations at free hapten concentrations of 10μM. It is apparent that γ chains from antibody populations usually bind the homologous hapten, but not an unrelated hapten, to a greater extent than do normal γ chains. The differences are

small and varied from one antibody population to another, but cannot be attributed to contaminating unsplit antibody: even at the upper limit of 2% contamination this would add an increment of only 0.02.

The variations in binding showed no obvious correlation with either the association constants for the hapten–(parent antibody) interaction or the hapten binding shown by the light chains (Table 3). Binding

Table 2. *Specificity of the binding of haptens by rabbit γ chains*

Binding was established by equilibrium dialysis against one or the other hapten at a free hapten concentration of 10 μM. The ranges given are standard deviations of quadruplicate samples dialysed simultaneously; all figures without a range are averages of duplicates or triplicates. Antisera from individual rabbits represented in the table were not included also in the pooled antisera.

Source of γ chains	Source of IgG (number of rabbits providing serum pool)	Binding of indicated hapten (mol/mol of γ chain dimer; mol.wt. 104000)	
		Methyl Orange	ε-Dnp-lysine
Normal IgG	12	0.20±0.026	0.09±0.011
Anti-ABS a	4	0.27	0.09
b	1	0.34	0.11
c	1	0.22	0.08
d	1	0.27	0.08
Anti-Dnp a	6	0.17	0.16
b	1	0.20	0.21
c	1	0.18	0.13

Table 3. *Comparison of binding of Methyl Orange by γ and light chains from three preparations of anti-ABS from individual rabbits*

Average association constants were determined as described in the Materials and Methods section. Binding by the chains was established by equilibrium dialysis against 10 μM-Methyl Orange.

Preparation	Binding of hapten by whole antibody: average association constant (l·mol)	Binding of hapten by isolated chains (mol/mol of chain dimer)	
		γ chains	Light chains
(a)	7×10^8	0.34	0.10
(b)	2.2×10^8	0.22	0.08
(c)	$>10^9$	0.27	0.08

Table 4. *Binding of Methyl Orange by normal and anti-ABS IgG preparations and their γ chains: dependence on hapten concentration*

In all cases binding was established by passage through columns of Sephadex G-25 (fine) at 4°C. The columns (1.8cm×20cm) were equilibrated with Methyl Orange at the indicated concentrations. Samples of protein (4ml; 1.5–2.0mg/ml) were applied to duplicate (IgG) or triplicate (γ chain) columns; the effluent was collected in lots of 3ml and extinctions were read at 280 and 465nm. The protein was delivered in 12–20min. The anti-ABS was from pooled antisera from 12 rabbits.

	Hapten bound (mol/mol of IgG or γ chain dimer)			
Concn. of free hapten (μM) ...	5	10	30	100
Normal IgG	<0.05	<0.05	0.06	0.12
Normal γ chains	0.11	0.18	0.42	0.77
Anti-ABS IgG	1.9	1.9	2.0	2.1
Anti-ABS γ chains	0.19	0.29	0.51	0.90

by γ chains from different preparations of normal IgG showed no significant differences.

Table 4 shows the dependence of binding on hapten concentration for IgG molecules and their γ chains. Again the smallness of differences between the normal and antibody γ chains is apparent. The antibody chains clearly have a much smaller average association constant for binding than do the parent molecules. Attempts to define the valency of the chains by an extrapolation to infinite hapten concentration, i.e. to the x axis of the Scatchard (1949) plot in Fig. 2, gave indefinite results: the flattening of the curve at higher hapten concentrations suggests that very weakly binding sites are beginning to make appreciable contributions (Scatchard *et al.*, 1957).

Binding by fragments of the γ chains

Table 5 shows that Fc fragments from normal and anti-ABS γ chains bound minute and equal amounts of Methyl Orange, whereas the Fd fragments showed molar bindings comparable with the whole γ chains.

Fig. 3 depicts the binding of Methyl Orange by Fd fragments from anti-ABS, found upon gel chromatography in the presence of 0.5% sodium deoxycholate. The Fd fragment appears to exist as a mixture of monomers and dimers, with the latter binding three times as much hapten as the former. Any allowance for contamination of the monomer peak with dimer and vice versa will increase the calculated disproportion in binding. Adding deoxycholate to 0.5% to solutions of whole γ chains did not disrupt the dimers.

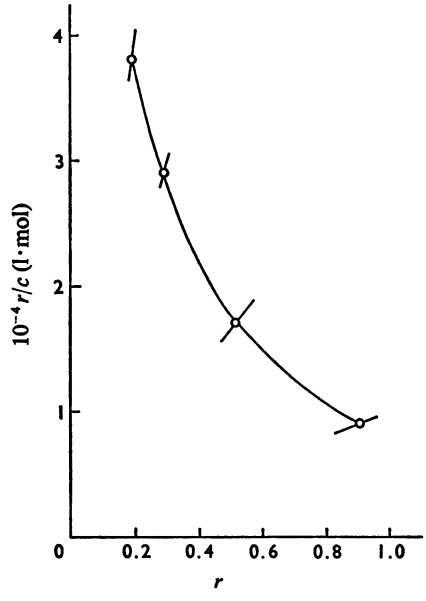

Fig. 2. *Binding of the homologous hapten Methyl Orange by the γ chains from rabbit anti-ABS, plotted by the method of Scatchard* (1949)

r is mol of bound hapten/mol of γ chain dimer, c the concentration of free hapten. Straight lines through the experimental points, directed towards the origin, represent the range of triplicate determinations.

Table 5. *Binding of Methyl Orange by the Fd and Fc fragments of normal and anti-ABS IgG preparations*

Binding was established by gel chromatography as described in Table 4. The anti-ABS was from the same pooled antiserum as in Table 4.

	Hapten bound (mol/mol of Fd dimer or Fc dimer)	
Concn. of free hapten (μM) ...	10	100
Normal Fd fragment	0.17	0.66
Normal Fc fragment	0.02	0.15
Anti-ABS Fd fragment	0.30	0.83
Anti-ABS Fc fragment	0.02	0.13

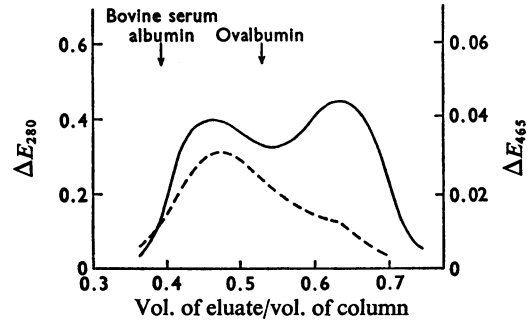

Fig. 3. *Binding of the homologous hapten Methyl Orange by Fd' fragments from rabbit anti-ABS in the presence of deoxycholate*

A Sephadex G-100 column (1.5cm×72cm) was equilibrated at 4°C with 10μM-Methyl Orange, 0.5% sodium deoxycholate, 4mM-sodium acetate buffer, pH5.4. Fd' fragments, 5mg in 5ml of equilibrating solution, were introduced. ——, ΔE_{280} (distribution of protein); ----, ΔE_{465} (distribution of bound hapten). The arrows indicate the positions of elution of the protein standards bovine serum albumin (mol.wt. 68000) and hen ovalbumin (mol.wt. 44500), measured at the points of inflexion of the leading edges. The two peaks given by Fd' fragments appear therefore to represent dimer and monomer. Calculated binding of hapten (mol/mol of dimer): at the height of the dimer peak, 0.22; at the height of the monomer peak, 0.075.

Purity and dispersity of the light chains

Light chains from both anti-Dnp and anti-ABS at 10mg/ml gave no reaction by the Ouchterlony technique with sheep antisera to Fd and Fc fragments of rabbit IgG, indicating that neither whole IgG nor Fab fragment could have been present in amounts exceeding 2% of the total protein.

It must be emphasized that the standard method of preparing these chains involved alkylation of those SH groups with a potential for forming interchain S–S links, leaving non-covalent bonds as the only interchain type available. Upon chromatography in Sephadex G-100 in a dissociating solution (4.0M-guanidine–HCl, 0.1M-sodium formate buffer, pH3.0), rabbit normal light chains were eluted as a symmetrical peak in the same position as human light chains. This confirms their similar molecular weights (about 23000) and rules out significant proteolytic degradation. In non-dissociating buffers within the range pH5.4–9.0 they sedimented in the ultracentrifuge as a single broad peak, with $s_{20,w}$ between 2.2 and 2.9S. Such values suggest a mixture of monomers and dimers. When examined by sedimentation equilibrium the normal chains gave a distribution of protein (Fig. 4) resembling that found by Stevenson & Dorrington (1970) for human light chains. There is a large arbitrary element in fitting the two straight lines to the plot in Fig. 4, but the apparent molecular weights calculated from the slopes are clearly consistent with the presence of both monomers and dimers. Finally the fact that these forms are interconverting, or largely interconverting, was demonstrated by the failure of all attempts to separate distinct monomeric and dimeric fractions by chromatography on Sephadex G-100 in neutral buffers.

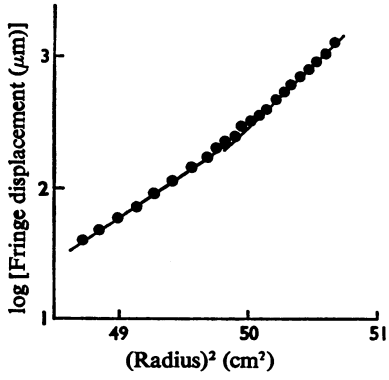

Fig. 4. *Sedimentation equilibrium of light chains from rabbit normal IgG in* 0.1M-*NaCl–4*mM-*sodium acetate buffer, pH5.4, after* 33*h at* 34000*rev./min*

Apparent molecular weights from the two fitted straight lines: top, 32000; bottom, 23000.

Light chains from purified anti-Dnp and anti-ABS gave the same results as did the normal chains when examined in the ultracentrifuge by sedimentation velocity. The presence of a homologous hapten (ε-Dnp-lysine and Methyl Orange respectively) at a concentration of 100μM made no observable difference.

Light chains with an interchain S–S link, examined in sodium acetate buffer, pH5.4, *I* 0.1, had a value for $s_{20,w} = 3.5_1$S. They gave by sedimentation equilibrium a linear plot for log (fringe displacement) against the square of radial distance and an apparent molecular weight of 49000. They were thus a monodisperse solution of dimers.

Binding of haptens by the light chains

Unless stated otherwise the results apply to preparations without an interchain S–S link. Extents of binding found upon equilibrium dialysis at pH8.0, *I* 0.1, and at three different concentrations of hapten are set out in Table 6. It can be seen that light chains from both antibodies exhibited low extents of binding, which reflect the specificities of the parent molecules. The amounts of binding at 30μM-hapten are some five times higher than could be accounted for by contamination of the light chains with whole antibody IgG or its Fab fragment. Essentially the same results were obtained upon dialysis at pH5.4, with *I* either 0.1 or 0.004.

Table 6. *Binding of haptens by antibody light chains*

Binding was established by equilibrium dialysis with the protein at a concentration of 2.0–5.0mg/ml.

	Hapten bound (mol/mol of light chain dimer)											
	ε-Dnp-lysine			2,4-Dinitrophenol			Methyl Orange			CTABS*		
Concn. of free hapten (μM) ...	10	30	100	10	30	100	10	30	100	10	30	100
Anti-Dnp light chains	0.05	0.11	0.14	0.05	0.09	0.11	0.01	0.02	0.05	<0.01	0.01	0.03
Anti-ABS light chains	<0.01	0.02	0.04	<0.01	<0.01	0.04	0.08	0.10	0.14	0.04	0.09	0.13

* 4-(*N*-Chloroacetyltyrosine-azo)benzene-1-sulphonate.

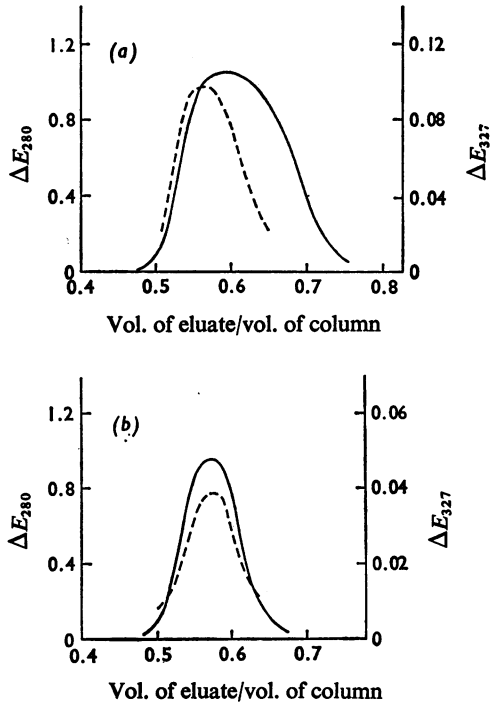

Fig. 5. *Elution patterns given by light chains from anti-ABS*

Light chains from anti-ABS were passed through a Sephadex G-100 column (1.6cm × 87cm) equilibrated with 10μM-4-(*N*-chloroacetyltyrosine-azo)benzene-1-sulphonate–0.1M-NaCl–4mM-sodium acetate buffer, pH5.4. The protein samples to be applied had earlier been dialysed for 48h at 4°C against the equilibrating solution. The extinctions plotted are those above the equilibrating solution: ——, ΔE_{280}, indicating the distribution of protein; ----, ΔE_{327}, indicating the distribution of bound hapten. (*a*) Light chains prepared by the standard method, with only non-covalent interchain bonds. Applied sample: 3ml, 4.2mg/ml. Molar binding per light chain dimer: at the height of the hapten peak, 0.21; overall, 0.11. (*b*) Light chains with an interchain S–S bond. Applied sample: 3ml, 2.2mg/ml. Overall molar binding per light chain dimer: 0.094.

When binding was established by passage through Sephadex G-100 equilibrated with homologous hapten there was a notable disparity in the distributions of protein and bound hapten in the effluent (Fig. 5*a*). The hapten peak was reached before the protein peak, and more than 70% of the bound hapten was located within the first half of the protein zone. The same result was obtained when light chains from anti-Dnp were put through a column equilibrated with 30μM-Dnp-lysine. No such disparity occurred when light chains stabilized in the dimeric form by S–S links were put through the same column (Fig. 5*b*).

Discussion

Both light chains and Fd fragments appear to dimerize via non-covalent bonds not present in the parent molecules. The light chain–light chain and Fd–Fd interactions might well involve those molecular areas participating in light chain–Fd fragment bonding in the parent IgG (Björk & Tanford, 1971). The apparent stability of γ-chain dimers could be due to their maintenance by both the Fc–Fc interactions which occur in IgG and the new Fd–Fd interactions.

Ultracentrifugal and chromatographic studies of the light chains have indicated a monomer–dimer mixture with at least a large part of the population undergoing rapid interconversion between the two forms. The distribution of bound hapten shown in Fig. 5(*a*) is explicable only in terms of its being restricted to that protein species with a faster than average mobility on the Sephadex column, that is to the dimers. This is readily visualized for any dimers which are stable. For interconverting dimers a detailed analysis would require more knowledge of both the dimerization and dimer–hapten interactions, but there seems no reason to doubt that transient dimers could account for most of the observed binding. The chromatogram in Fig. 5(*b*) demonstrates the coincidence of protein and bound hapten peaks when monodisperse light chains are transported in this system. It failed to show an increase in the extent of binding due to stabilization of the dimeric state. However, there might have been included among these dimers a variety of aberrant products resulting from the oxidation of free SH groups, particularly if intrachain S–S bonds were involved to any extent in an overall disulphide-exchange process.

Painter *et al.* (1972) observed better binding among a light chain population regarded as monomeric than among one regarded as dimeric. However, they point out that the 'monomeric' population possibly consisted largely of dimers at the concentrations used for binding studies, and leave the stoicheiometry of binding undecided.

The relative amounts of hapten bound by γ chains and Fd' and Fc fragments reveal that binding of hapten by the first of these is dependent mainly upon its *N*-terminal (Fd) half. In accord with the demonstration that antigen–antibody reactions are little affected by the presence of 0.5% deoxycholate (Crumpton & Parkhouse, 1972), dimers of Fd' in the presence of this detergent bound over 70% of the amount of hapten bound in buffer alone; monomers, in contrast, bound relatively little. This result implies

that, as with the light chains, the main binding site for hapten in Fd' and by extension in γ chains depends upon the dimeric state. Painter *et al.* (1972) reached the same conclusion after examining the stoicheiometry of binding.

The random element involved in forming binding sites by dimerization implies that the efficacy of hapten-binding by any given pair of γ or light chains will be largely fortuitous: it will depend upon the ways in which half-sites, well matched in the parent molecules, re-sort themselves. It is quite easy to visualize extreme situations where the hapten simply will not fit into a new site, or where it fits so well as to give an association constant similar to that in the parent antibody. Under these circumstances it is not to be expected that binding by isolated chains could give reliable quantitative indications of their original contributions to antibody sites. The limited findings in Table 3 support this conclusion. No simple treatment of the binding data for the γ and light chains of the three antibody populations represented will yield the average association constants for the parent antibodies. From their results with a pooled anti-Dnp population Painter *et al.* (1972) calculated that the unitary free energies of binding for isolated γ and light chains summed to give the free energy for binding by intact antibody. It is difficult to visualize the molecular basis for this finding. The argument does not seem to take account of the fact that if the chain sites are dimeric then one γ chain site and one light chain site together contain the constituent parts of two antibody sites, not one.

A feature of the present results has been the smallness of the specific incremental binding shown by the antibody γ chains above that shown by their normal counterparts. The relatively high binding by the normal chains seems to be the main feature distinguishing γ chain from light chain binding. Some reports imply that it might not be unusual for a binding site to appear rather catholic at our working concentrations of free hapten. Thus once one starts measuring binding due to association constants of 2×10^4 l·mol or less one can find binding of haptens, apparently at the antibody sites, by irrelevant IgG (Parker & Osterland, 1970). Glazer (1970) has also commented on the prevalence of 'non-specific' binding at the specific binding sites of globular proteins. Altogether the fact that the γ chains bind more hapten than the light chains, found by many previous workers and confirmed here, must be seen in the contexts of the dimeric binding sites, of the γ chains being wholly instead of partially dimeric, and of the higher background given by normal γ chains.

A final question is whether antibody-like activity among chain dimers has any physiological relevance. In man free light chains circulate in extracellular fluid and account for more than 50% of urinary immunoglobulin (reviewed by Berggård & Peterson, 1967). They appear to represent a synthetic overspill (Stevenson, 1962; Vaughan *et al.*, 1967) and it is doubtful that they exhibit any significant antibody activity (Turner & Rowe, 1969). However, another conceivable role is as cell-surface immunoglobulin. Among a mass of conflicting evidence about the nature of the receptor for antigen on T lymphocytes (e.g. Crone *et al.*, 1972) are experiments suggesting the presence of light chains but of no known heavy chains on the cell surfaces (Mason & Warner, 1970; Greaves *et al.*, 1971; Rouse & Warner, 1972). The experiments reported in this paper demonstrate that an antibody site formed by light chains alone is at least feasible.

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