The Recombination of Dimers of Immunoglobulin Peptide Chains

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1. Both the γ and light peptide chains of human pooled and myeloma immunoglobulin G can be prepared as non-aggregating dimers at pH 5.4 in 4 mM-sodium acetate buffer. The dimeric state is maintained by non-covalent bonds, since the formation of interchain disulphide bonds was prevented by alkylation of the thiol groups. In the case of the light chains there is some evidence that the dimers are in equilibrium with a small amount of monomer. 2. When such dimers of the γ and light chains are mixed at pH 5.4 in 4 mM-sodium acetate buffer they combine rapidly, yielding a product that resembles the original immunoglobulin G in its physicochemical and antigenic properties. However, the original optical rotatory dispersion spectrum was regained only with the homogeneous myeloma protein. The recombined pooled immunoglobulin G had a spectrum slightly different from the original, suggesting that at least some of the recombinant molecules had not regained native conformations. 3. Dimers of γ chains stabilized by interchain disulphide bonds were able to recombine with light chains. However, light chains stabilized in the dimeric state by interchain disulphide bonds would not combine with γ chains. 4. The chains of rabbit immunoglobulin G behave similarly to the human chains in this system, apart from the alkylated light chains showing clearer evidence of monomeric components.

The nomenclature used in this paper is that recommended by the World Health Organization (1964).

Each molecule of mammalian IgG† consists of two heavy (more specifically, γ) and two light peptide chains. The chains can be dissociated by reducing the interchain disulphide bonds at neutral pH and by then transferring the protein to a suitable dissociating solvent, such as *m*-propionic acid, to disrupt the non-covalent interchain bonds (Fleischman, Pain & Porter, 1962). In the acid the light chains exist as monomers (Gally & Edelman, 1964), though the dispersity of the γ chains is in doubt.

If a mixture of γ and light chains in propionic acid is transferred by dialysis into a neutral buffer a large measure of recombination occurs to give molecules resembling the original IgG (Olins & Edelman, 1964; Roholt, Onoue & Pressman, 1964). It would be desirable to avoid the need for dialysis, to study, for example, the kinetics or the optical

correlates of recombination (Dorrington, Zarlengo & Tanford, 1967; Genco, Karush & Tenenhouse, 1968). However, the separate introduction of peptide chains into neutral buffers, for recombination when they are mixed, has been precluded by aggregation of the γ chains. An attempt has been made to prevent this by introducing multiple poly-DL-alanyl groups into the IgG (Fuchs & Sela, 1965). The present paper describes a method for keeping the γ chains monodisperse, as dimers, in a buffer suitable for recombination, without the need for chemical modification. When the γ and light chains in this buffer are mixed there is a rapid net reaction: $\gamma_2 + \text{light}_2 \rightarrow \gamma_2\text{light}_2$. A preliminary account of this work has been published (Stevenson, 1968a).

MATERIALS AND METHODS

Dithiothreitol (Cleland's reagent, A grade) was obtained from Calbiochem, Los Angeles, Calif., U.S.A. Iodoacetamide was recrystallized from ethanol. Guanidine hydrochloride was prepared by neutralizing commercial guanidine carbonate, previously recrystallized from ethanol, with conc. HCl. The guanidine hydrochloride was further recrystallized from methanol.

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† Abbreviations: IgG, immunoglobulin G; o.r.d., optical rotatory dispersion.

Pooled human IgG was obtained from Commonwealth Serum Laboratories (Melbourne, Vic., Australia) in the form of Cohn's fraction II-1,2. Human myeloma IgG (Go) was isolated from the serum of a patient with multiple myeloma. Rabbit IgG was prepared from pooled normal plasma. The myeloma protein and rabbit IgG were precipitated twice with 1.6M-(NH₄)₂SO₄ at pH 8.0, dissolved in and dialysed against 0.03M-sodium phosphate buffer, pH 7.3, and then passed through a column of DEAE-cellulose (Whatman DE32) equilibrated with this buffer. All IgG preparations were freed of aggregate by passage through Sephadex G-150 equilibrated with 0.2M-tris-HCl buffer, pH 8.0.

The preparation of alkylated γ and light chains was the same for both human and rabbit proteins, and was based on the procedure of Fleischman *et al.* (1962). IgG (2mg/ml of 0.2M-tris-HCl buffer, pH 8.0) was allowed to react with 0.01M-dithiothreitol for 30 min at room temperature. Iodoacetamide (0.22M in the same buffer) was then added in a molar amount 1.1 times that of the thiol groups, and allowed to react for 10 min at room temperature. The reaction was stopped by passing the protein through Sephadex G-25 (coarse grade) equilibrated with *m*-propionic acid. Finally the γ and light chains were separated by passage through Sephadex G-100 in *m*-propionic acid at room temperature; the ratio of column volume to protein was approx. 10 cm³/mg. The entire preparation up to this stage was completed within 24 h. The yield of light chains, as measured by E_{280} in the propionic acid, for all the IgG preparations was 27% of the recovered material. The γ chains eluted as a single peak with a skewed leading edge. The use of more concentrated protein solutions or prolongation of the exposure to propionic acid was apt to cause the γ chains to appear as a double or more complex set of peaks, the leading part of which apparently represented aggregate.

The γ and light chains were transferred separately into 4mM-sodium acetate buffer, pH 5.4, by dialysis in the cold room. This buffer replaces that of pH 5.0 used previously (Stevenson, 1968a). An earlier method of transfer into buffer, by passage through Sephadex G-25, was abandoned because of appreciable binding of protein to the Sephadex at low ionic strength. The γ chains were obtained finally at a concentration of about 0.5 mg/ml and the light chains at about 0.2 mg/ml. These solutions were concentrated by ultrafiltration through 8/32 in cellophan tubing (Visking) under a pressure of 700 mmHg (IgG and γ chains) or 560 mmHg (light chains).

Protein concentrations were measured spectrophotometrically by using the following values for $E_{1\text{cm}}^{1\%}$ at 280 nm: IgG, 13.5; γ chains, 14.0; light chains, 12.0.

Ultracentrifugal analyses were performed with two Beckman-Spinco model E ultracentrifuges both equipped with RTIC temperature control units and one with an electronic speed control. Molecular weights were determined by using the meniscus depletion method of Yphantis (1964) as described by Montgomery, Dorrington & Rockey (1969). Interference optics were used as a routine. For concentration and speed-dependence studies cells with six-channel centrepieces and sapphire windows were used. The partial specific volumes of γ and light chains were taken as 0.74 and 0.718 ml/g respectively in the 4mM-acetate buffer. In 6.0M-guanidine-HCl the effective $\bar{v}$ of γ chain was taken as 0.725 to allow for the

preferential binding of guanidine (for discussion see Montgomery *et al.* 1969). The density of the 4mM-acetate buffer was assumed to be the same as that of water while that of 6.0M-guanidine-HCl was measured pycnometrically (1.14 g/ml). Sedimentation coefficients were corrected to the viscosity of water at 20°C by using the data of Svedberg & Pedersen (1940), any very small correction for the viscosity and density of the acetate buffer being ignored.

Electrophoresis in horizontal slabs of polyacrylamide gel was carried out as described by Stevenson & Straus (1968).

O.r.d. spectra were obtained with a ORD/UV-5 spectropolarimeter (Japan Spectroscopic Co.) equipped with a thermostated cell-holder maintained at $25 \pm 0.05^\circ\text{C}$, between 210 and 300 nm. Cylindrical cells (Helma, Westcliff-on-Sea, Essex, U.K.) of light-paths 5.0, 2.0, 1.0 and 0.1 mm were used with protein concentrations between 0.5 and 1.5 mg/ml. Each protein solution was examined at a minimum of two concentrations and in two cells of different light-path to eliminate the possibility of cell or stray-light artifacts. The results are expressed in terms of the reduced mean residue rotation, $[m']$, at each wavelength obtained from the specific rotation $[\alpha]_\lambda$ using the following relationship:

$$[m']_\lambda = [\alpha]_\lambda \frac{3M_0}{100(n^2 + 2)}$$

where M_0 is the mean residue weight, taken as 110 for IgG and its peptide chains, and n is the refractive index of the solvent.

RESULTS

Dispersity and molecular size of the peptide chains from human IgG in 4mM-sodium acetate buffer, pH 5.4. Provided that the preparative schedule outlined above was adhered to closely, γ chains

Fig. 1. Elution patterns given by γ and light chains on being passed through a column (3.3 cm $\times$ 75 cm) of Sephadex G-100. The starting and equilibrating buffer was 4mM-sodium acetate, pH 5.4. Applied volumes and concentrations were: γ chains, 10 ml, 1 mg/ml; light chains, 10 ml, 1.7 mg/ml. Elution volumes were measured at the points of inflexion of the leading edges and are indicated. It was important to prime the column by putting through some of the corresponding protein before each definitive run to saturate the binding sites (Stevenson, 1968b).

Fig. 2. Human γ chains, 5 mg/ml in 4 mM-sodium acetate buffer, pH 5.4, examined by sedimentation velocity in the ultracentrifuge. Conditions were: speed, 59780 rev./min; temperature, 20°C; photograph, 53 min after reaching full speed; phase-plate angle, 60°. $s_{20,w}$ was found to be 4.0 S. In this and subsequent ultracentrifugal patterns sedimentation was from the left.

Fig. 3. Dependence of $s_{20,w}$ on protein concentration for human IgG ($\circ$), recombined IgG (Δ) and γ chains ($\square$). Native IgG and recombined IgG at the same or similar concentrations were examined simultaneously by the use of standard and wedge-window cells, thereby eliminating differences that might have resulted from variations in temperature and rotor speed. For the γ chains, $s_{20,w} = 5.24$ S.

stored at about 1 mg/ml in the acetate buffer for up to 3 weeks in the cold appeared monodisperse when examined by gel chromatography on Sephadex G-100 (Fig. 1). Their elution position in this system lay between those of IgG and bovine serum albumin, suggesting dimers. Intact IgG gave an elution profile of the same shape.

When concentrated to 5 mg/ml and examined by sedimentation velocity in the ultracentrifuge the γ chains revealed a small amount of aggregate,

whereas the rest of the protein sedimented as a single boundary with $s_{20,w}$ 4.0 S (Fig. 2). The promotion of aggregation at higher ionic strength could be readily demonstrated when solid sodium chloride was added to the γ chain solution (final concn. 0.1 M) immediately before the centrifuge run. Under such conditions 60–70% of the protein appeared as a fast-moving rapidly spreading peak with an average sedimentation coefficient near 17 S. The concentration-dependence of $s_{20,w}$ for γ chain in the 4 mM-sodium acetate buffer gave no indication of dissociation into smaller units at the lowest concentrations (Fig. 3). There was quite a marked dependence of $s_{20,w}$ on concentration, presumably due to the incomplete suppression of the primary charge effect (Svedberg & Pedersen, 1940) in the buffer of low ionic strength, yielding $s_{20,w} = 5.24$ S.

Sedimentation equilibrium of γ chains in the 4 mM-sodium acetate buffer gave values for the apparent weight-average molecular weight (M_w) that showed a marked dependence on initial protein concentration and, to a lesser extent, on rotor speed (Fig. 4b). The concentration-dependence was assumed to be due to the primary charge effect previously seen in sedimentation-velocity studies. The dependence on speed was presumed to be due to the presence of small amounts of aggregated γ chain, although this could not be detected in the plots of $\log$ (fringe displacement) versus square of radial distance (Fig. 4a). When extrapolating the M_w versus concentration curve to obtain M_w^0 , values obtained at the higher rotor speeds were used. The validity of this decision was confirmed when γ chains were examined in 6.0 M-guanidine-hydrochloric acid at various initial concentrations and rotor speeds (Figs. 4a and b). Little or no dependence of M_w on either of these factors was apparent and all the results were used to calculate a value for M_w^0 in 6.0 M-guanidine-hydrochloric acid of 51800 g/mol. This value clearly indicates that γ chains exist as dimers, molecular weight near 104000, in the 4 mM-acetate buffer.

The migration rate and elution profile given by alkylated light chains on Sephadex G-100 are shown in Fig. 1 and the schlieren pattern of their sedimenting boundary is shown in Fig. 12. Aggregation was notably absent but was often observed in preparations that were not alkylated. Sedimentation in the ultracentrifuge suggested that the chains were present entirely as dimers, because values of $s_{20,w}$ were similar to those given in this buffer by disulphide-stabilized dimers of pathological light chains (Bence-Jones proteins). None of these examinations revealed any tendency to dissociate into smaller units. However, when subjected to sedimentation equilibrium the light chains exhibited marked curvature in the plots of $\log$ (fringe displacement) versus square of radial distance,

Fig. 4. (a) Sedimentation equilibrium of human γ chains in 4 mM-sodium acetate buffer, pH 5.4 (right), and 6.0 M-guanidine-HCl (left) after 24 and 45 h respectively at rotor speeds of 24 000 and 30 000 rev./min respectively. Initial protein concentration was 0.5 mg/ml. The vertical line, r_b , represents the radial position of the solution-fluorocarbon interface. (b) Rotor speed and concentration dependence of the weight-average molecular weights of human γ chains in 4 mM-sodium acetate buffer, pH 5.4 ($\square$ and $\blacksquare$) and 6.0 M-guanidine-HCl ($\circ$ and $\bullet$). Rotor speeds were (rev./min): $\blacksquare$, 18 000; $\square$, 24 000; $\bullet$, 26 000; $\circ$, 30 000. For further explanation see the text.

Fig. 5. Sedimentation equilibrium of human light chains in 4 mM-sodium acetate buffer, pH 5.4, after 30 h at 36 000 rev./min. Bottom, $M = 24\,000$; Top, $M = 39\,800$.

indicating mass heterogeneity. When the centrifuge was run at a speed appropriate for monomeric light chain (i.e. mol.wt. of 23 000) and at a high

initial protein concentration (1.0 mg/ml) the equilibrium distribution of protein showed two distinct components (Fig. 5). The more centripetal slope yielded a molecular weight near 24 000 whereas the heavier component had a molecular weight near 40 000. Clearly these results do not provide an unambiguous molecular weight for the two components but do suggest that the light-chain dimer preparations do contain a species with a molecular weight consistent with light-chain monomer.

Recombination of the chains. This was done with alkylated chains, except where stated otherwise. When concentrated solutions of γ and light chains in the acetate buffer were mixed in equimolar amounts and examined immediately in the ultracentrifuge the result shown in Fig. 6(a) was obtained. The bulk of the protein was now represented by a species with a sedimentation coefficient the same as that of native IgG, which had appeared at a rate too high to be followed in this type of experiment. This new species is regarded as recombined IgG. Accompanying it were small amounts of leading and trailing material; the former is presumed to be aggregate and the latter free light chains. In most experiments it was convenient to mix the chains in unconcentrated solutions, i.e. with the γ chains initially at about 0.5 mg/ml and the light chains at about 0.2 mg/ml. With the light chains present in twofold or greater molar excess the yield of recombined IgG, in terms of

utilization of the γ chains, was close to 100%. With the light chains present in only an equimolar proportion an appreciable amount of aggregate was often encountered during the purification procedures.

Purification of the recombined product was achieved by ultrafiltration to a protein concentration of 5–10mg/ml, dialysis against 0.02M-tris-hydrochloric acid buffer, pH 8.0, and passage through Sephadex G-150 equilibrated with the same buffer. The concentration and transfer to a neutral or mildly alkaline buffer, whose composition is not critical, tends to aggregate and precipitate any uncombined γ chains, and at the new pH and ionic strength the binding of protein to Sephadex, which is troublesome in the presence of the low ionic strength acetate buffer (Stevenson, 1968b), was not observed. Further measures to remove uncombined γ chains and other aggregated material (Stevenson, 1968a) were found unnecessary in later work.

Characterization of recombined IgG. The purified recombined product remained monodisperse when stored in neutral buffers in the cold (Fig. 6b). The value of $s_{20,w}$ and its dependence on protein concentration were the same as for native IgG (Fig. 3). Both proteins exhibited the same range of mobilities when subjected to electrophoresis at pH 8.0 in 4% polyacrylamide gel. Light-chain contents of 24 and 26%, as measured by E_{280} in propionic acid, were found for two samples of recombined IgG separated into their constituent chains by passage through Sephadex G-100 in M-propionic acid; the

corresponding value for native IgG is 27%. A comparison of the antigenic structures of the native and recombined proteins by the Ouchterlony method revealed no differences (Fig. 7). The antiserum used to obtain the pattern in Fig. 7 usually gave two precipitin lines with pooled IgG, and it was shown by allowing it to react simultaneously with Bence-Jones proteins that one line was due to κ -containing and the other to λ -containing IgG. The fact that two distinct lines were given by the recombined IgG suggests that this consists predominantly of κ - or λ -containing molecules, and that few molecules can contain both types of light chains.

O.r.d. spectra of native and recombined human IgG, γ and light chains. Both the human pooled IgG and myeloma IgG gave qualitatively similar o.r.d. spectra between 220 and 270nm (Fig. 8). Both spectra are characterized by a small amount of rotation throughout the wavelength range, Cotton effect minima near 230 and 225nm, and a small negative Cotton effect centred near 240nm. Quantitatively the spectra show considerable differences. This variation in the amounts of rotation is a characteristic feature of the o.r.d. spectra of all immunoglobulin classes so far studied (Steiner & Lowey, 1966; Dorrington & Tanford, 1968; Dorrington & Rockey, 1968). The γ chains from both the IgG samples show a greater laevorotation than the parent IgG with a clearly defined minimum at 230–231nm and poorly resolved shoulders near 240 and 225nm (Fig. 8). The light chains derived from both IgG samples showed only a single Cotton-effect minimum at 225nm and again the amount of rotation was greater than for the parent proteins throughout the wavelength range (Fig. 8). The peptide chains showed differences in laevorotation

Fig. 6. Human recombined IgG examined by sedimentation velocity in the ultracentrifuge. (a) Solutions in 4mM-sodium acetate buffer, pH 5.4, of γ chains, 7.8mg/ml, and light chains, 6.2mg/ml, were mixed in equimolar proportions at room temperature and examined immediately in the ultracentrifuge. Conditions were: speed, 55070 rev./min; temperature, 20°C; photograph, 45 min after reaching full speed and 64 min after mixing; phase-plate angle, 65°. $s_{20,w}$ was found to be 5.6S. (b) Recombined IgG purified as described in the text. Conditions were: protein concentration, 6.0mg/ml; buffer, 0.02M-tris-HCl, pH 8.0; speed 52000 rev./min; temperature, 20°C; phase-plate angle, 70°; photograph, 40 min after, reaching full speed. $s_{20,w}$ was found to be 6.3S.

Fig. 7. Comparison of the antigenic structures of human native IgG and recombined IgG on an Ouchterlony plate. The unlabelled well contains rabbit antiserum to native IgG. This is seen to have reacted with native (N) IgG and recombined (R) IgG to give a reaction of complete antigenic identity. The significance of the double lines is discussed in the text.

Fig. 8. O.r.d. spectra, between 220 and 270nm, of (a) human myeloma IgG and (b) human pooled IgG and their γ and light-chain dimers in 4mM-sodium acetate buffer, pH 5.4.

Fig. 9. O.r.d. spectra, between 220 and 270nm, of native and recombined (a) human myeloma IgG and (b) human pooled IgG in 4mM-sodium acetate buffer, pH 5.4. The third curve (H+L) represents a theoretical IgG spectrum based on the weighted sum of the o.r.d. spectra of the separated γ and light chains shown in Fig. 8.

apparently correlated with the rotation of the parent IgG, although the differences between the γ chains was more pronounced than between the light chains.

The o.r.d. spectrum of purified recombined

human myeloma IgG was qualitatively and quantitatively indistinguishable from that of the native protein, within experimental error (Fig. 9). Also shown in Fig. 9 is the theoretical o.r.d. spectrum for an equimolar mixture of γ and light chains calcu-

lated from the spectra of the isolated chains according to the relationship:

$$[m']_{\lambda}, \text{IgG} = 0.70 [m']_{\lambda}, \gamma + 0.30 [m']_{\lambda}, \text{light}$$

taking into account the weight-proportions of the chains in the intact IgG. Clearly the o.r.d. spectrum of IgG is not a simple addition of contributions from each type of peptide chain. Similar studies on the pooled human IgG showed that the o.r.d. spectrum of the purified recombined protein was quantitatively different from the parent IgG although it showed similar qualitative features (Fig. 9). As with the myeloma protein the spectrum of the recombined pooled IgG was different from that obtained by appropriate addition of the peptide chain spectra (Fig. 9). Analogous studies with pooled rabbit IgG yielded results essentially similar to those obtained with pooled human IgG.

Participation of disulphide-bonded dimers. Disulphide-bonded dimers of light chains were obtained as described by Gally & Edelman (1964). First the chains were prepared without alkylating the thiol groups released by reduction of disulphide bonds. They were then concentrated to 1.5 mg/ml and dialysed for 3 days against 0.1 M-tris-hydrochloric acid buffer, pH 8.0, to encourage the oxidation of thiol groups. The disulphide-bonded dimers could then be separated from the remainder by passage through Sephadex G-100 in M-propionic

acid, since only the former persist as dimers in the acid. About 80% of the chains fell into this category. They were transferred by dialysis into the acetate buffer and tested for their ability to combine with a normal (alkylated) preparation of γ chains. No combination was apparent on examination in the ultracentrifuge (Fig. 10).

Disulphide-bonded dimers of the γ chains were obtained similarly, with modifications due to their tendency to aggregate. Again the chains were prepared with omission of alkylation. They were transferred by dialysis into the acetate buffer in the usual way, and then were dialysed for 24 h at 3°C against the acetate buffer saturated with oxygen. Despite the fact that pH 5.4 is not favourable for oxidation of thiol groups (Cecil & McPhee, 1959) it was found that more than 90% of the γ chain dimers became disulphide-bonded. Under these conditions it was judged unnecessary to separate the disulphide-bonded species from the remainder. The extent of bonding was assessed by passing a sample through a column of Sephadex G-100 in 4 M-guanidine-hydrochloric acid-0.1 M-sodium formate buffer, pH 3.0 (Fig. 11). The disulphide-bonded dimers of γ chains showed a completely unimpaired ability to recombine with alkylated light chains in the acetate buffer.

Fig. 10. Simultaneous examination by sedimentation velocity in the ultracentrifuge of human alkylated γ -chain dimers (a) (in a wedge-window cell) and the same plus an equimolar amount of disulphide-stabilized light-chain dimers (b) (in a standard cell). Total protein concentrations were: in the wedge-window cell, 4.0 mg/ml; in the standard cell, 6.4 mg/ml. Conditions were: speed, 59780 rev./min; temperature, 23°C; phase-plate angle, 55°; photograph, 80 min after reaching full speed. Values of $s_{20,w}$ for the main peaks are: in the wedge-window cell, 4.65S; in the standard cell, 4.3S. The main peak in the standard cell undoubtedly represents free γ chains and the trailing shoulder free light chains; there can be little if any recombined IgG present. The lower value of $s_{20,w}$ for the γ chains in the standard cell is attributable to their sedimenting in an environment of higher protein concentration.

Fig. 11. Elution patterns given by human alkylated (—) and non-alkylated (----) γ chains on being passed through an upward-flowing column (2.5 cm $\times$ 89 cm) of Sephadex G-100 equilibrated with 4 M-guanidine-HCl-0.1 M-sodium formate, pH 3.0. The proteins had been kept in 4 M-sodium acetate buffer, pH 5.4 (see the text) and immediately before application to the column were dialysed for 16 h at room temperature against 6 M-guanidine-HCl-0.1 M-sodium formate, pH 3.0. In each case 8 ml of sample, at a protein concentration of approx. 1 mg/ml, was applied to the column and followed by 10 ml of 6 M-guanidine-HCl and then by 4 M-guanidine-HCl. The low pH value was used to prevent disulphide interchange and oxidation of free thiol groups in the guanidine solution. The non-alkylated chains were eluted predominantly in a position consistent with dimers, accountable only by the presence of interchain disulphide bonds.

Fig. 12. Alkylated light chains in 4mM-sodium acetate buffer, pH 5.4, examined by sedimentation velocity in the ultracentrifuge: (a), human, 8 mg/ml; (b), rabbit, 6 mg/ml. Conditions were: speed, 59780 rev./min; temperature, 20°C; phase-plate angle, (a) 70°, (b) 55°; photograph, (a), 96 min, (b), 110 min after reaching full speed. The values of $s_{20,w}$ are (a) 3.2S, (b) 2.8S.

Application to rabbit IgG. Much of the foregoing has been found to apply equally well to rabbit IgG and its peptide chains: the γ chains were obtained as a monodisperse solution of dimers in the acetate buffer, and they recombined promptly with light chains to give a product indistinguishable from native IgG by all the tests described apart from o.r.d. (see above). However, the light chains, when examined in the ultracentrifuge, gave a broad sedimenting peak skewed on its trailing side, with a value of $s_{20,w}$ notably less than that of the human chains (Fig. 12). The same sample, when examined by gel chromatography on Sephadex G-100 in 4.0M-guanidine-hydrochloric acid-0.1M-formate buffer, pH 3.0, eluted as a symmetrical peak in the same position as human light chains; thus it seems not to have undergone significant proteolytic degradation. All attempts to separate the light chains into two species by gel chromatography in the acetate buffer failed. It appears, therefore, that rabbit alkylated light chains exist in the acetate buffer as a mixture of interconverting monomers and dimers, with a much greater proportion of monomers than is observed among the human light chains.

DISCUSSION

The present paper describes a method for the preparation of the γ and light chains from IgG that has two distinct advantages over methods available hitherto. First, the γ chain is obtained as a monodisperse dimer virtually free of aggregated material. This has permitted detailed physicochemical measurements on the γ chains, and direct comparisons of the isolated chains and intact IgG. Secondly, recombination of the chains, by the overall reaction $\gamma_2 + \text{light}_2 \rightarrow \gamma_2\text{light}_2$, takes place on mixing the chains in the acetate buffer, without the

necessity of mixing in a dissociating solvent before dialysis into a neutral buffer. This single-solvent recombination should permit kinetic measurements to be made. By using such kinetic measurements and the associated thermodynamic parameters a great deal of information might be obtainable regarding the specificity of γ -light chain interactions which have been demonstrated in myeloma proteins and purified antibodies.

The nature of the buffer used in this study was dictated by the need to keep the γ chains monodisperse over the concentration range required for the characterization by chromatographic and physicochemical methods. At lower protein concentrations recombination of the γ and light chains might well occur in buffers of more physiological pH and ionic strength.

In the acetate buffer the γ chains had a molecular weight near 104 000 which dropped to half this value in 6.0M-guanidine-hydrochloric acid, clearly indicating that they exist as dimers in the first buffer. The molecular weight and the value of 5.24S for $s_{20,w}^0$ can be used to calculate the frictional ratio ($f/f_{\min.}$) of the γ_2 species (Tanford, 1961), which provides an estimate of its deviation from a compact globular structure. The value obtained, $f/f_{\min.} = 1.44$, is outside the range for typical globular proteins (1.10–1.25) but is close to the value calculated for intact IgG (1.47). It is clear therefore that γ_2 , like intact IgG, does not behave as a typical globular protein, indicating that it possesses either considerable asymmetry or a large amount of hydration. It should be remembered that in the sense used here 'hydration' includes hydrodynamically trapped solvent, so that a molecule with some regions of flexibly coiled polypeptide chain would appear to have an anomalously large amount of bound solvent. This is particularly relevant since current models for IgG propose that the γ chains are made up of compact globular regions connected by an extended, flexible 'hinge' region (for full discussion see Dorrington & Tanford, 1970). The similarity in the hydrodynamic properties between IgG and the γ dimer is particularly satisfying since the γ chains in intact IgG form a dimeric structure.

The o.r.d. studies have been used as a probe for conformational differences between the isolated peptide chains and native IgG as well as a measure of recovery of native conformation following recombination. It is established that o.r.d. can give information regarding conformational transitions but interpretation of such effects in terms of specific changes in secondary and tertiary folding is fraught with difficulties (Dorrington & Tanford, 1970). It is clear, from the present studies, that the conformation of intact IgG is not simply an addition of the conformations of the constituent peptide chains.

This must mean that recombination of γ and light chains is accompanied by a conformational change, and in the case of the myeloma IgG the conformation of the recombined protein is indistinguishable from that of the native IgG. Recombination of chains derived from pooled human (or rabbit) IgG did not give products with an average conformation identical with that of the native protein. It must be stressed that the difference in conformation might be quite small and not incompatible with the apparent hydrodynamic and antigenic similarity of the two proteins. These observations are similar to those published earlier by Dorrington *et al.* (1967) using the conventional method for chain recombination. In that study the laevorotations of the γ chain and of the recombined pooled IgG were greater than reported here, probably owing to aggregation of the γ chain and some incomplete recombination. Dorrington *et al.* (1967) also failed to detect the Cotton effect at 240nm in the γ chain and the recombined pooled IgG but found it to be present in the recombined myeloma IgG. They suggested that this spectral property acted as a marker for the recovery of the native conformation. However, the present results clearly show that the 240nm Cotton effect is associated with the γ chain only and can be seen both in the recombined pooled IgG and myeloma IgG.

The significance of the failure to recover the native average conformation of pooled human IgG following recombination under conditions where myeloma IgG shows complete recovery has been discussed (Dorrington *et al.* 1967; Dorrington & Tanford, 1970). The myeloma protein consists of a homogeneous population of molecules, each with the same amino acid sequence, so that random recombination of the chains gives molecules chemically indistinguishable from each other and from the native IgG. In pooled IgG, however, there is a heterogeneous mixture of molecules with different antibody specificities and amino acid sequences. Recombination of chains in this case will result in molecules made up predominantly of chains derived from different parent IgG molecules. The o.r.d. data suggest that at least some such 'hybrid' molecules have conformations differing significantly from those of the original IgG molecules which have their chains 'correctly' paired at biosynthesis.

From the o.r.d. characteristics of the γ and light chains described here and from data on the Fab and Fc fragments (Steiner & Lowey, 1966) it is possible to account for the o.r.d. characteristics of IgG in terms of its constituent peptide chains. The 230nm Cotton effect is due to the C-terminal halves of the γ chains since Fc fragment has its single Cotton effect at this wavelength. The 240nm Cotton effect is located in the N-terminal region of the γ chain (Fd) since it is not shown either by

light chain or Fc fragment but is apparent in isolated γ chain and Fab fragment. The 225nm minimum is contributed to by both Fd and light chain since Fab has a minimum at this wavelength. It is noteworthy that the spectral differences between native and recombined IgG are greatest in those regions associated with either both γ and light chains (225nm minimum) or the N-terminal region of the γ chain (240nm Cotton effect).

If the recombined IgG we obtained is structurally similar to the native molecule, as is suggested by physicochemical and antigenic characterization, then the reaction leading to its formation is unlikely to involve a simple juxtaposition of γ_2 and light₂. The non-covalent forces maintaining the dimeric state of the light chains with notable specificity (Stevenson & Straus, 1968) appear not to exist in intact IgG molecules, because Fab fragments, retaining the conformation possessed in intact IgG (Goodman & Gross, 1963; Steiner & Lowey, 1966) and each containing one entire light chain (Fleischman, Porter & Press, 1963), show no tendency to dimerize. Presumably, the formation of recombined IgG is accompanied by rupture of the light-light non-covalent bonds. Whether this rupture occurs before or after the formation of the species γ_2 -light₂ is not known. The former possibility, restated, implies that the recombination funnels through monomeric light chain:

We have presented evidence that the light chains exist as monomers to a small extent in the acetate buffer. However this does not constitute proof that all light chain dimers can convert readily into monomers in this buffer in order to react with γ chains. Studies of Bence-Jones proteins (Bernier & Putnam, 1963; Deutsch, 1965) indicate that some light chains occur in monomeric form to a much greater extent than others.

The complete failure of disulphide-stabilized dimers of light chains to combine with dimers of γ chains does add further weight to the possibility that recombination has to funnel through monomeric light chain. The presence of a disulphide bond between the two light chains is not by itself incompatible with the existence of the species $\gamma_2 \text{ light}_2$ (Grey, 1969).

Evidence has been obtained from studies of mouse plasmacytomas *in vitro* that monomers of light chains occur intracellularly as an intermediate synthetic product (Askonas & Williamson, 1966; Schubert, 1968). However, the characterizations of dispersity have involved the presence of deoxycholate, or of urea and dodecyl sulphate, where

non-covalent interchain bonds are unlikely to survive. It can be inferred from these studies only that no disulphide-stabilized dimers of light chains were detectable in the cellular extracts.

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