Recovery of Native Conformation of Rabbit Immunoglobulin G upon Recombination of Separately Renatured Heavy and Light Chains at Near-Neutral pH*

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ABSTRACT: Recombination of separated, renatured heavy and light chains from rabbit immunoglobulin G has been shown to occur successfully at near neutral pH; reassociation of the chains under these conditions has not been previously reported, apart from one report for human immunoglobulin by Stevenson. The purified reconstituted protein was found to be identical with native immunoglobulin by immunological and hydrodynamic measurements, and also by optical rotary dispersion and circular dichroism analyses. The latter result differs from earlier studies in this laboratory, in which a difference in conformation between native and reconstituted nonspecific immunoglobulin was found. This difference led to the suggestion that authentic immunoglobulin G molecules can be formed only when heavy chains combine with uniquely complementary light chains. On the basis of the present paper there is no evidence for such obligatory specificity.

The heavy and light chains of rabbit immunoglobulin G (IgG)[1] are readily separated from each other in 1 M propionic acid, where they exist as monomeric, denatured polypeptide chains (Fleischmann *et al.*, 1962). The two preceding papers (Bjork and Tanford, 1971a,b) have shown that each separated chain can be renatured by removal of propionic acid and adjustment to near-neutral pH. In this paper we report the recombination of the separately renatured chains, at near-neutral pH, to give a product that is indistinguishable from native IgG by all criteria applied. This result is of interest for several reasons. (1) Although reconstitution of IgG from separated heavy and light chains has been reported by several authors (Franêk and Nezlin, 1963; Olins and Edelman, 1964; Metzger and Mannik, 1964; Haber and Richards, 1966), reassociation was always carried out by mixing the separated chains in their monomeric, denatured form. The renaturation process was then performed on the mixture of chains. Because the separately renatured chains differ in conformation from the conformations which they possess when combined with each other in the native IgG molecule, and because they have interactions that could well prevent recombination, such as the interaction between the Fd portions of two heavy chains in the renatured heavy-chain dimer, it was by no means certain that reconstitution at near-neutral pH would occur at all. (After this manuscript was completed, we were made aware of a paper by Stevenson (1968), showing that stable dimers of both heavy and light chains of human IgG can be obtained under conditions similar to those employed by us for rabbit IgG, and that they can be recombined at near-neutral pH to form a stable reconstituted IgG molecule, with physical properties close to those of native IgG. More detailed physical measurements are provided in a subsequent paper by Stevenson and Dorrington (1970). They report that similar results were obtained for rabbit IgG, but do not provide detailed data. Recombination of polyalanylated chains of rabbit IgG at neutral pH (after reduction and reoxidation of intrachain disulfide bonds) has been demonstrated by Freedman and Sela (1966).) (2) It has been previously reported from this laboratory (Dorrington *et al.*, 1967) that reconstitution of fully authentic native IgG occurs only for homogeneous preparations, such as myeloma IgG, but not for a heterogeneous preparation, such as the nonspecific serum IgG used in this study. This conclusion will be shown to be incorrect. When the chains are prepared as described in the two preceding papers, all properties of the reconstituted IgG, including optical rotatory properties, are indistinguishable from those of the parent native protein. (3) The preceding paper on renaturation of separated light chains (Björk and Tanford, 1971b) shows that there are two kinds of light chain in rabbit IgG, one of which exists in the renatured state as a dimer, the other as a monomer. It is of interest to compare the ability of these two species to recombine with the heterogeneous mixture of heavy chains.

Materials and Methods

Purification of rabbit IgG and separation of its heavy and light chains by gel chromatography in 1 M propionic acid were carried out as described in a preceding paper (Björk and Tanford, 1971a).

Recombination of IgG from heavy and light chains was performed in two different ways. In the first procedure solutions of separated heavy and light chains in 1 M propionic acid were adjusted to a protein concentration of 0.5 mg/ml and were mixed in molar ratios (H:L) of 1:1 or 1:2 while still in propionic acid. Each of these mixtures (about 100 ml) was dialyzed against three changes of 2000 ml of 0.01 M sodium acetate buffer (pH 5.5), followed by 2000 ml of 0.02 M sodium acetate buffer (pH 5.5), containing 0.1 M NaCl. The solutions were then concentrated by ultrafiltration

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[1] The nomenclature of and the abbreviations for the immunoglobulins and their subunits produced by reduction and proteolysis are those recommended by the World Health Organization (1964).

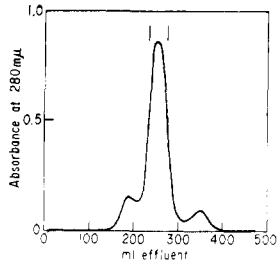

FIGURE 1: Purification on Sephadex G-200 in 0.02 M sodium acetate buffer (pH 5.5)–0.1 M NaCl of IgG, reconstituted from heavy and light chains during dialysis from 1 M propionic acid. 41 mg of protein in 6 ml of solution was applied to a 2.5 × 90 cm column. The vertical lines indicate those fractions used for further analyses.

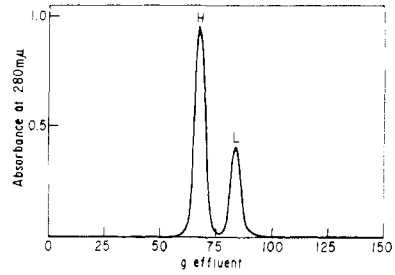

FIGURE 2: Separation of heavy and light chains of fully reduced and carboxymethylated, reconstituted IgG on a 1.5 × 85 cm column of 6% agarose in 6 M Gdn·HCl. About 5 mg of protein in 0.2 ml of solution was applied to the column. The effluent emerging from the column was determined by weight instead of by volume.

through a UM20E membrane (Amicon Corp., Lexington, Mass.) to a volume of 6 ml, *i.e.*, to a protein concentration of about 8 mg/ml, and were applied to a column (2.5 × 90 cm) of Sephadex G-200 in 0.02 M sodium acetate buffer (pH 5.5)–0.1 M NaCl. The elution rate of this column was 20 ml/hr and 5-ml fractions were collected. The purified, reconstituted protein was concentrated by ultrafiltration as above and was then studied.

In the second procedure, recombination was carried out at pH 5.5, after the isolated heavy and light chains had been renatured separately. Heavy chain was renatured by extensive dialysis against 0.01 M sodium acetate buffer (pH 5.5) and light chain by extensive dialysis against 0.02 M sodium phosphate buffer (pH 7.0), containing 0.1 M NaCl. Light-chain monomer and dimer were separated by gel chromatography on Sephadex G-75, as described previously (Björk and Tanford, 1971b) and were then dialyzed against 0.01 M sodium acetate buffer (pH 5.5). Solutions of renatured heavy chains and either light-chain monomers or dimers, all at pH 5.5, were adjusted to a protein concentration of 0.8 mg/ml and were mixed in molar ratios (H:L) of 1:1 or 1:2. The mixtures, each containing 50 mg of protein were allowed to stand at 5° for 20 or 70 hr and were then reduced by ultrafiltration to a volume of about 5 ml, *i.e.*, to a protein concentration of about 10 mg/ml. This solution was dialyzed for 8–10 hr against 0.02 M sodium acetate buffer (pH 5.5)–0.1 M NaCl; during this procedure a small amount of precipitate (2–5% of the total amount of protein present) was formed and was removed by centrifugation. The supernatant was applied to the same G-200 column as was used in the first recombination procedure, and the column was eluted in an identical manner. The resulting purified, recombined IgG was concentrated by ultrafiltration and investigated.

All analytical procedures have been thoroughly described in the two preceeding papers (Björk and Tanford, 1971a,b). All measurements were carried out at 25°.

Results

Reconstitution of IgG from Heavy and Light Chains Mixed in 1 M Propionic Acid. Recombination by dialysis from propionic acid was carried out mainly to compare IgG, reconstituted in this commonly employed manner, with IgG, reconstituted at near-neutral pH from separately renatured chains, which will be described below. The procedure used in these experiments was essentially that described by Metzger and Mannik (1964), *i.e.*, the chains were mixed in propionic acid and the acid was removed by dialysis against a buffer of pH 5.5 and low ionic strength. This procedure minimizes aggregation of heavy chains, which is more pronounced at higher pH and ionic strength (Björk and Tanford, 1971a). Even under these optimal conditions, however, a certain amount of heavy-chain polymerizes, and the recombined IgG thus must be purified. Figure 1 shows a typical result of a gel chromatographic separation on Sephadex G-200 of a recombination mixture with a 1:1 molar ratio of heavy to light chain. One major peak, consisting of recombined IgG, is seen. The first small peak appears at the position expected for aggregated heavy chain (Björk and Tanford, 1971a), and the last peak elutes as free light chain.

When an excess of light chain was present in the recombination mixture, the heavy-chain aggregate peak disappeared, but, of course, the light-chain fraction increased in amount. The recoveries in all these gel chromatographic experiments were 94–98%.

Purified, recombined IgG has been found to be indistinguishable from native IgG in hydrodynamic and antigenic properties by several investigators, using essentially the same recombination technique (separated chains mixed in propionic acid before renaturation) as described above (Olins and Edelman, 1964; Roholt *et al.*, 1964; Fougereau and Edelman, 1964; Metzger and Mannik, 1964). For this reason only a few confirmatory experiments of this kind have been performed in order to ascertain that the recombined IgG obtained is similar to that described by other authors. Immunodiffusion and immunoelectrophoresis against goat antirabbit IgG showed only one precipitin band, identical with both native and mildly reduced and carboxymethylated IgG. In sedimentation velocity experiments only one component was seen, having a sedimentation coefficient at 8 mg/ml of 6.5 S. This value is similar to that of native IgG at the same concentration, which is 6.4 S (Noelken *et al.*, 1965). The proportions of heavy and light chains in the recombined immunoglobulin were determined by gel chromatography of the fully reduced and carboxymethylated protein on 6% agarose in 6 M guanidine hydrochloride (Gdn. HCl[2]) (Fish *et al.*, 1969). This procedure causes a complete dissociation of the chains and allows a quantitative separation of them (Figure 2). The recombined IgG was found to contain 32–33% by weight of light chain and, correspondingly, 67–68% of heavy chain. These values have been corrected for the difference between the extinction coefficients of the two chains. The value for the light-chain content of native IgG, based on molecular weights of 23,000 and 50,000, is 31.5%. The observed proportions agree well with this.

[2] Abbreviation used is: Gdn·HCl, guanidine hydrochloride (this is not the preferred abbreviation of the authors).

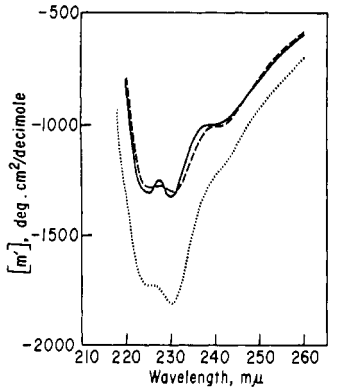

FIGURE 3: Optical rotatory dispersion spectrum of IgG, reconstituted from rabbit heavy and light chains by dialysis from 1 M propionic acid (broken line) and of native rabbit IgG (solid line). The solvent was 0.1 M NaCl (pH 5.5) and protein concentrations were 0.5 mg/ml. The dotted line represents the curve calculated for an equimolar mixture of heavy and light chains, as described in text.

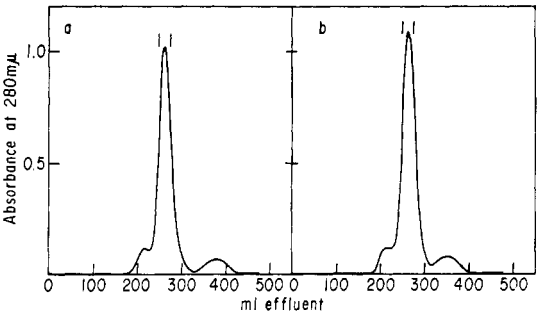

FIGURE 4: Purification on Sephadex G-200 in 0.02 M sodium acetate buffer (pH 5.5)–0.1 M NaCl of IgG reconstituted at pH 5.5 from separately renatured chains. (a) Heavy-chain dimer and light-chain monomer. (b) Heavy-chain dimer and light-chain dimer; 45 mg of protein applied to a 2.5 × 90 cm column. The vertical lines indicate those fractions used for further analyses.

The conformation of IgG, reconstituted in the classical manner, has been studied by Dorrington *et al.* (1967). Contrary to this report we find that, in addition to all other properties investigated, the optical rotatory dispersion spectrum of nonspecific rabbit IgG, reconstituted in this way and subsequently purified, is also identical with the native IgG spectrum within experimental error (Figure 3). The curve calculated for an equimolar mixture of heavy and light chains from data in previous papers (Björk and Tanford, 1971a,b) is given for comparison. Since unfractionated light chains were used for these recombination experiments, the calculations were based on a mixture of 60% light-chain monomers and 40% dimers (Björk and Tanford, 1971b). This was done because the two forms have somewhat different optical rotatory dispersion spectra. The weighted average of the heavy- and light-chain curves is quite different from the spectrum of the native protein, whereas the spectra of native and recombined IgG are identical. The curves thus show that chain separation of nonspecific rabbit IgG is accompanied by a conformational change that is reversible within the limits detectable by optical rotatory dispersion, provided uncombined chains are removed. Reversibility without purification was shown for myeloma proteins by Dorrington *et al.* (1967), but in their investigation the native optical rotatory dispersion spectrum of nonspecific rabbit and human IgG was not fully regained. Because these authors did not purify their recombined material, however, their results can be explained by the assumption that aggregation of heavy chain was insignificant during recombination of myeloma IgG, but more pronounced when nonspecific IgG was recombined.

Reconstitution of IgG at pH 5.5 from Separately Renatured Heavy and Light Chains. Recombination of the chains in this manner has not been previously reported in the literature, and a detailed study of the recombined IgG thus obtained was therefore undertaken. In this recombination procedure the heavy and light chains were renatured separately in the way described in previous papers (Björk and Tanford, 1971a,b). The solvent used for the recombination experiments was one of low ionic strength and pH, *i.e.*, 0.01 M sodium acetate buffer (pH 5.5) in which very little aggregation of heavy chains occurs, and the gel chromatographic purification of these chains on Sephadex G-200 was therefore omitted. The renatured light-chain preparation was separated into its monomer and dimer fractions and these were dialyzed against 0.01 M sodium acetate buffer (pH 5.5). Recombinations were carried out by mixing heavy chains, which exists as a dimer at near-neutral pH, with either light-chain monomer or dimer using molar ratios of heavy to light chains of 1:1 or, in some experiments, 1:2. The mixture was usually kept at 5° for a total of 30 hr before unreacted chains were removed. During the last 10 hr of this period the ionic strength of the sample was raised by dialysis against 0.02 M sodium acetate buffer (pH 5.5)–0.1 M NaCl. Removal of remaining free heavy and light chains was accomplished as in the first recombination procedure by gel chromatography on Sephadex G-200 (Figure 4). The two curves shown are those obtained in experiments in which heavy chain was recombined with light-chain monomer (Figure 4a) and with light-chain dimer (Figure 4b), in both cases using a 1:1 molar ratio of the two chains, and allowing recombination to occur for 30 hr before application of the mixture to the column. As in the first recombination procedure, two small peaks, one containing aggregated heavy chains and the other free light chains, are seen in both experiments, in addition to the main fraction, which is shown below to consist of recombined IgG. The recoveries in these and all similar chromatographies performed were 93–97%. In order to ascertain that maximum recombination had taken place, the recombination mixture was kept for 80 hr in a few experiments, before the uncombined chains were removed. Results identical with those presented in Figure 4 were obtained, indicating that equilibrium is reached in less than 30 hr. No investigations of the kinetics of the recombination process have been performed, however. An excess of light chains (a molar ratio of 1:2) did not result in the disappearance of the fraction containing heavy-chain aggregates, in contrast to recombination taking place during dialysis from propionic acid.

The protein in both of the two main gel chromatographic fractions in Figure 4 was extensively investigated. Unless specifically indicated, all studies reported below performed with material, purified after about 30-hr incubation from a mixture containing heavy and light chains in a 1:1 molar ratio. In all analyses undertaken, the protein in either of the two peaks was shown to be very similar or identical with native IgG, indicating that these peaks contain reconstituted IgG and that complete recombination of heavy and light chains had occurred also in this recombination procedure.

An immunodiffusion experiment against goat antirabbit IgG of recombinants obtained from heavy-chain and light-chain monomer or dimer is presented in Figure 5. The lines

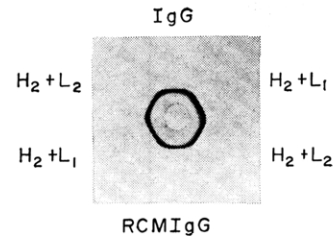

FIGURE 5: Immunodiffusion of native IgG, mildly reduced and carboxymethylated IgG (RCMIgG) and IgG reconstituted at pH 5.5 from heavy-chain dimer (H_2) and either light-chain monomer (L_1) or dimer (L_2). The antiserum was goat antirabbit IgG and protein concentrations were 1.0 mg/ml.

of identity show that all of the antigenic determinants present in native IgG were regained in both cases on recombination of the dissociated molecule. Since the antiserum recognized determinants associated with both the Fab and the Fc regions of the native molecule, the results indicate that the antigenic structure of both these regions has been fully regained.

The proportions between heavy and light chains in the recombined proteins were analyzed as described earlier by gel chromatography of the fully reduced and carboxymethylated proteins on an agarose column in 6 M Gdn·HCl, and the results are given in Table I. In this table are also included results for recombined material obtained both after a longer incubation time and with excess light chain present. As before the values presented have been corrected for the difference between the extinction coefficients of the two chains. The experimentally determined proportions agree very well with the values calculated for native IgG, indicating that recombined IgG also contains equimolar amounts of heavy and light chains. This holds true even when an excess of light chains is used in the recombination experiments.

Molecular weights of IgG, reconstituted at near-neutral pH, were determined in meniscus depletion sedimentation equilibrium experiments at different speeds (Table II). Values ranging from 139,000 to 145,000 were obtained, regardless of whether light-chain monomer or dimer was used for the recombinations. These values are identical,

TABLE I: Proportions between Heavy and Light Chains in IgG, Recombined at pH 5.5 from Heavy-Chain Dimer (H_2) and Either Light-Chain Monomer (L_1) or Dimer (L_2).

	Molar Ratio (H:L) in Mixture	Incubn Time (hr)	Weight %[a]	
			L Chain	H Chain
$H_2 + L_1$	1:2	80	31	69
	1:1	80	33	67
	1:1	30	32	68
$H_2 + L_2$	1:2	80	31	69
	1:1	80	33	67
	1:1	30	31	69

[a] The values for native IgG, based on molecular weights for heavy and light chains of 50,000 and 23,000, are 68.5% H and 31.5% L.

TABLE II: Molecular Weights, Determined by Sedimentation Equilibrium at 25°, of IgG, Recombined at pH 5.5 from Heavy-Chain Dimer (H_2) and Either Light-Chain Monomer (L_1) or Dimer (L_2).[a]

	Speed (rpm)	Mol Wt
$H_2 + L_1$	14,290	142,000
	15,220	143,000
	17,250	139,000
$H_2 + L_2$	14,290	142,000
	15,220	144,000
	17,250	145,000

[a] The solvent was 0.02 M sodium acetate buffer (pH 5.5), containing 0.1 M NaCl, and protein concentrations were 0.20–0.25 mg/ml.

within experimental error, with published molecular weights for rabbit IgG (Cammack, 1962; Marler *et al.*, 1964; Noelken *et al.*, 1965; Small and Lamm, 1966). Plots of the logarithm of fringe displacement *vs.* the square of the distance from the center of rotation are shown in Figure 6; they are linear from displacements of 50–70 μ, thus indicating homogeneity of the samples.

The shape of IgG, reconstituted from separated chains at pH 5.5, is also very similar to that of native IgG, as indicated by sedimentation velocity experiments. Sedimentation coefficients are plotted *vs.* protein concentration in Figure 7; no difference is seen between values determined for the two reconstituted immunoglobulins that are obtained by combination of heavy chain with either light-chain monomer or dimer. The data extrapolate to a sedimentation constant at zero protein concentration of 6.66 ± 0.04 S, a value in excellent agreement with sedimentation constants reported for native rabbit IgG, which range from 6.5 to 6.7 S (Charlwood, 1959; Cammack, 1962; Noelken *et al.*, 1965). Only one peak was seen in all sedimentation velocity experiments, which means that the samples contained insignificant amounts of impurities.

The conformations of native IgG and IgG reconstituted at near-neutral pH were shown to be identical, as judged from optical rotary dispersion and circular dichroism measurements. The optical rotatory dispersion curves obtained are presented in Figure 8 and the circular dichroism spectra in Figure 9. In both these figures the curves calculated for an equimolar mixture of heavy-chain and either light-chain monomer or dimer are given for comparative purposes. These curves show that there is a considerable conformational difference between native IgG and its separated *renatured* heavy and light chains. They are the curves that would have been measured if this conformational change had not been reversible on recombination of the chains. As can be seen, however, complete reversibility of the optical rotatory dispersion and circular dichroism curves, within experimental error, is obtained both when light-chain monomer and light-chain dimer is recombined with heavy chain. This is more clearly demonstrated by the optical rotatory dispersion measurements, since in circular dichroism the differences between the calculated and measured curves are smaller, and also the experimental errors are somewhat larger.

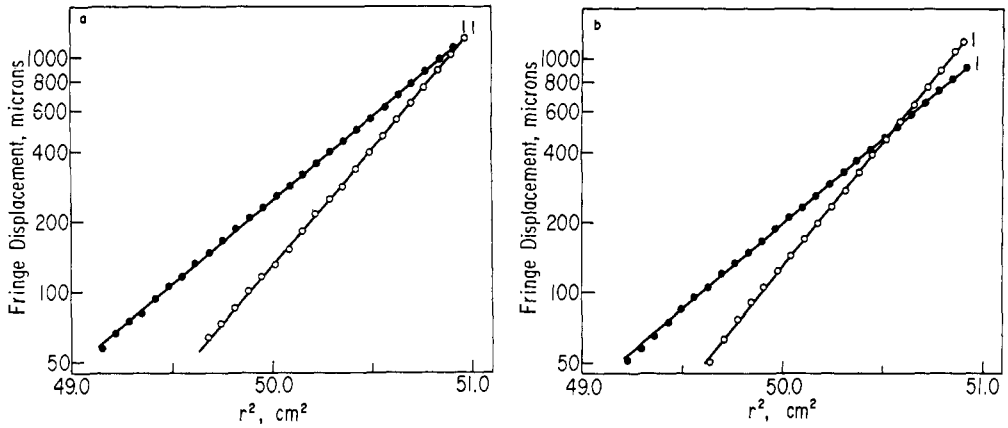

FIGURE 6: Meniscus depletion sedimentation equilibrium of IgG, reconstituted at pH 5.5 from heavy-chain dimer and (a) light-chain monomer, (b) light-chain dimer. Filled circles 14,290 rpm; open circles 17,250 rpm. The solvent was 0.02 M sodium acetate buffer (pH 5.5)–0.1 M NaCl, and protein concentrations were 0.2–0.25 mg/ml. The vertical lines indicate the cell bottoms.

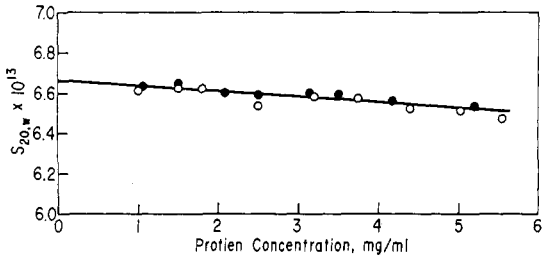

FIGURE 7: Sedimentation coefficients at different protein concentrations of IgG, reconstituted at pH 5.5 from heavy-chain dimer and light-chain monomer (open circles) or light-chain dimer (filled circles). The solvent was 0.02 M sodium acetate buffer (pH 5.5)–0.1 M NaCl.

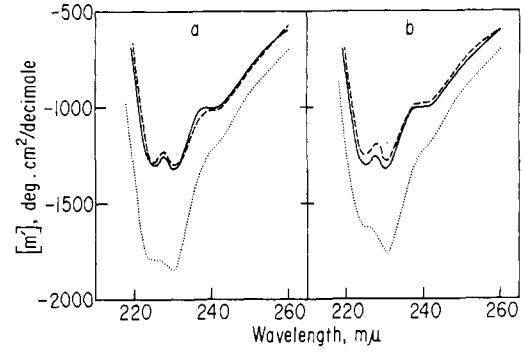

FIGURE 8: Optical rotatory dispersion spectra of native IgG (solid line) and IgG reconstituted at pH 5.5 (broken line) from heavy-chain dimer and (a) light-chain monomer, (b) light-chain dimer. The solvent was 0.1 M NaCl (pH 5.5) and protein concentrations were 0.5–0.55 mg/ml. The dotted lines represent the curves calculated for equimolar mixtures of heavy-chain dimer and either light-chain monomer or dimer.

Discussion

It is well established that separated heavy and light chains of IgG from several species can be recombined to form a reconstituted molecule, which is identical with native IgG in all properties reflecting gross conformation and chain arrangement (Olins and Edelman, 1964; Roholt *et al.*, 1964; Fougereau and Edelman, 1964; Metzger and Mannik, 1964). These reported recombination experiments have all been carried out in a similar way, that is the separated chains were mixed in propionic acid and recombination was allowed to take place during dialysis of this mixture to pH values around neutrality. The use of this recombination procedure probably was prompted by the notion that monomeric, and possibly partially unfolded, chains are the species involved in the recombination reaction. The latter part of this assumption has actually been strengthened by the discovery that heavy and light chains have conformations in free form different from those they possess when part of IgG (Dorrington *et al.*, 1967; Bjork and Tanford, 1971a,b). While this concept thus may be partially true, the investigations reported in this paper show that mixing of the chains in organic acid solution at low pH prior to recombination is not necessary; combination of chains takes place to a comparable extent even when separately renatured rabbit heavy and light chains are mixed at pH 5.5. As was noted in the Introduction, the same conclusion has been reached by Stevenson (1968) for human IgG. The mechanism of recombination may of course still involve monomeric light chains, and separation of heavy chains in the Fd region, and some unfolding and refolding either before or after association, but presumably sufficient concentrations of the requisite reactive forms exist in equilibrium with the predominant forms of the renatured chains. The existence of an equilibrium between the native state of a protein and a number of altered and disordered states (albeit with a very low equilibrium constant), even under native-like conditions, is probably a rather general phenomenon in protein chemistry (Tanford, 1970).

Similarly to what has been demonstrated for the commonly employed recombination procedure both in this and several other papers, reconstitution of rabbit IgG at near-neutral pH from separated heavy and light chains also results in a molecule, which is indistinguishable from native IgG in size, shape, chain proportions, and antigenic properties. Its molecular weight, measured by sedimentation equilibrium, was found to be 140,000–145,000 and its sedimentation constant 6.7 S, both properties in good agreement with those of native IgG. The proportion between heavy and light chains was identical with that of native IgG, and in immunodiffusion experiments recombined and native IgG showed immunological identity. From these experiments it is evident that the polypeptide chains of recombined IgG are arranged in the same general manner as in native IgG.

In addition to this similarity in gross arrangement of the

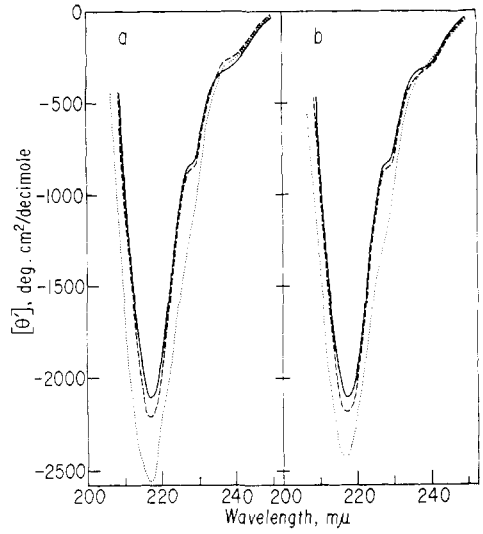

FIGURE 9: Circular dichroism spectra of native IgG (solid line) and IgG, reconstituted at pH 5.5 (broken line) from heavy-chain dimer and (a) light-chain monomer, (b) light-chain dimer. The solvent was 0.1 M NaCl (pH 5.5) and protein concentrations were 0.3–0.55 mg/ml. The dotted lines represent the curves calculated for equimolar mixtures of heavy-chain dimer and either light-chain monomer or dimer.

chains, investigations by optical rotatory dispersion and circular dichroism showed that the internal folding of the chains of recombined rabbit IgG also is identical with that of the chains of native IgG, within the limits detectable by these optical methods. This was shown both for IgG, reconstituted at near-neutral pH, and for IgG, reconstituted during dialysis from propionic acid. These results are in contrast to studies by Dorrington *et al.* (1967) which seemed to indicate that the native optical rotatory dispersion spectrum of nonspecific human and rabbit IgG was not fully regained on recombination of the chains in the classical manner. As was stated earlier, this observation, however, most probably was due to the fact that uncombined chains were not removed prior to measurements. It should be noted, however, that the human IgG reconstituted by Stevenson and Dorrington (1970), which appears to have comparable purity to ours by other criteria, possesses an optical rotatory dispersion spectrum retaining significant differences from that of native IgG.

A sequel to our results, demonstrating complete reversibility of the optical rotatory dispersion and circular dichroism spectra of nonspecific, heterogeneous IgG is the realization that the speculations by Dorrington *et al.* (1967), amplified by Tanford (1968), concerning the roles of interactions between complementary heavy and light chains in the generation of antibody specificity, may be largely incorrect, at least at the present stage of our knowledge. They were based on results supposedly showing full reversibility of conformation only upon combination of homologous chains from myeloma proteins, and not when heterologous chains in a heterogeneous immunoglobulin preparation were recombined.

It is of course well established that scrambling of the light and heavy chains of a heterogeneous antibody preparation leads to diminution of the free energy of association between antibody and antigen, so that the reconstituted IgG molecules prepared from separated heavy and light chains of nonspecific rabbit IgG cannot be identical in all respects with the original native preparation. But there is no evidence on the basis of the present studies that some of the reconstituted molecules lack a major conformational feature that is characteristic of all authentic native IgG molecules. The apparent lack of such a feature in our earlier work suggested that a given heavy chain could form an authentic IgG molecule (as defined by physical measurements) only with a restricted fraction of all available light chains. The present work shows that, within the sensitivity of optical rotatory dispersion and circular dichroism measurements in the detection of conformational differences, there are in fact no restrictions in the association of heavy and light chains. It is to be noted in particular that IgG molecules identical with native by all criteria we have used were obtained both when unfractionated heavy chains were recombined exclusively with monomeric and exclusively with dimeric light chains, although in the original IgG about 60% of the heavy chains were combined with the monomeric type of light chain and 40% with the dimeric type.

The observation that free native heavy and light chains can combine at pH values around neutrality to form an authentic immunoglobulin molecule may be of some importance in connection with certain aspects of the biosynthesis of immunoglobulins. Several experiments suggest that heavy and light chains are synthesized separately, and that the light chains are made at a higher rate and therefore enter an intracellular pool (Askonas and Williamson, 1966; Shapiro *et al.*, 1966; Williamson and Askonas, 1967; Schubert, 1968). Light chains from this pool combine with nascent or newly synthesized heavy chains, possibly while the latter are still attached to the polysomes (Shapiro *et al.*, 1966; Schubert and Cohn, 1968). Our results demonstrate the feasibility of such a combination between heavy and light chains at pH values near the intracellular value. Thus no intermediate, partially unfolded state of either chain has to be postulated to explain the combination of the two chains during assembly of the immunoglobulin molecule. Some special assumption would have been necessary if recombination of separated chains had been shown to occur *in vitro* only after the chains had been mixed in acid solution.

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Characterization and Chemical Modifications of Toxins Isolated from the Venoms of the Sea Snake, *Laticauda semifasciata*, from Philippines*

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ABSTRACT: The venom of *Laticauda semifasciata* from the Philippines, contains two toxins. These toxins were isolated and purified by means of Sephadex gel filtration and CM-cellulose column chromatography. The purity of the toxins was established using isoelectric focusing, electrophoresis, sedimentation velocity, and sedimentation equilibrium. Both toxins could be isolated in crystalline form. The lethality of the purified toxins increased five- to sixfold when compared to the original venom. The molecular weight as determined by amino acid composition, gel filtration, and by sedimentation equilibrium was approximately 6800 for both toxins. The amino acid compositions of the two toxins are quite similar.

Toxin a contains 62 amino acid residues and toxin b contains 61 amino acid residues. No free sulfhydryl groups were detectable. End-group analysis showed both toxins to be a single polypeptide chain with arginine at the N terminal and aspartic acid (asparagine) at the C terminal. The isoelectric points were determined by isoelectric focusing to be 9.15 for toxin a and 9.34 for toxin b. The toxicity of the toxins was unaltered by heating to 100° for 30 min or by exposure to pH extremes from 1 to 11. The toxicity was completely lost when the tryptophan residue in the purified toxins was modified with *N*-bromosuccinimide. However, no change in antigenicity was observed for the *N*-bromosuccinimide-modified toxins. No significant change in the toxicity was observed when the lysine and arginine residues of the purified toxins were modified.

The venoms of all sea snakes (*Hydrophiidae*) are extremely toxic (Tu, 1961; Tu and Ganthavorn, 1969; Homma *et al.*, 1964). These venoms are believed to act upon the neuromuscular junction of the victims (Rogers, 1902, 1903; Fraser and Elliott, 1905; Carrey and Wright, 1960a,b, 1961). The neurotoxic factors of sea snake venoms were first identified as protein by Arai *et al.* (1964). The isolation and preliminary characterization of some neurotoxic proteins from the venom of the sea snake, *Laticauda semifasciata* (*Erabu unagi*), from Japan have been described by Uwatoko *et al.* (1966a,b) and Tamiya *et al.* (1966). Enzymes, such as phospholipase A (Carrey and Wright, 1960a; Barme, 1958; Tu *et al.*, 1970; Setognchi and Ohbo, 1969), anti-coagulase (Barme, 1958; Cesaru and Boquet, 1936), L-leucyl-β-naphthylamide-hydrolyzing enzyme (Tu and Toom, 1967), and hyaluronidase (Barme and Detrait, 1959) have also been observed. In this paper, the isolation, purification, and the physicochemical and chemical characterization of two neurotoxic proteins from *Laticauda semifasciata* captured in the Philippines in the South China Sea are presented. In addition, we present some data concerning selective chemical modification of the tryptophan residue, lysine residues, and arginine residues. The antigenicity of *N*-bromosuccinimide-modified toxins is compared to that of the original toxins.

Materials

4-Mercuribenzoic acid, was purchased from Aldrich Chemical Co. Glutathione was obtained from the Nutritional Biochemicals Corp. Bacitracin, cytochrome *c* (horse heart), myoglobin (crystallized salt free, sperm whale), chymotrypsinogen A (beef pancreas, six-times crystallized, salt free), ovalbumin (two-times crystallized), trypsin were purchased as molecular weight markers (kit 8109) from Mann Research Laboratories. Blue Dextran, Sephadex G-10, and G-50 were obtained from Pharmacia Fine Chemicals, Inc; Cellex-CM from Bio-Rad; and Cellulose casing from the Union Carbide Corp. *N*-Bromosuccinimide, *O*-methylisourea, urea, guanidium chloride, and 1,2-cyclohexandione were obtained from the Aldrich Chemical Co. *N*-Bromosuccinimide was

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