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Gross Conformation of Free Polypeptide Chains from Rabbit Immunoglobulin G. II. Light Chain*

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ABSTRACT: Rabbit light chains were isolated in the usual manner by gel chromatography in 1 M propionic acid. The preparation thus obtained, when returned to neutral pH, consisted of apparently stable monomers and dimers and these were separated from each other by gel chromatography. The existence of monomeric and dimeric forms of rabbit light chains most probably is a result of intrinsic properties of two different classes of light-chain species. The monomer dimerizes reversibly at high protein concentrations, whereas a corresponding dissociation of the dimer form at low protein concentrations was not detected. Hydrodynamic parameters, such as frictional ratio and intrinsic viscosity, indicate that both forms deviate somewhat from a compact globular shape and that the dimer is slightly more extended than the monomer.

These results, and the cleavage of light chains into halves by proteolytic digestion, earlier reported for free human chains and confirmed here for rabbit light chains, lend some support to the proposed "domain" theory for the structure of immunoglobulins. A difference between the conformations of the monomer and dimer forms was deduced from optical rotatory dispersions and circular dichroism measurements, but it was not decided whether this difference is due to a conformational change accompanying dimerization or merely reflects different conformations of the two classes of light-chain species.

Investigations of the size, shape, and conformation of separated heavy and light chains of immunoglobulin G[1] are of great importance because of the significant contributions such studies can give to the elucidation of the relationship between structure and function of antibodies. In a companion paper (Björk and Tanford, 1971a) some of these physicochemical properties of isolated rabbit heavy chain were reported, and in this communication we describe similar properties of free rabbit light chain. As in the study of heavy chains, rabbit IgG was chosen as the parent protein, because of our ultimate aim to attempt to correlate the physicochemical properties of free chains from rabbit antibodies with their antigen-binding ability. The investigations show that some light-chain species exist in free form as apparently stable monomers and others as stable dimers, and that both forms have somewhat more extended shapes than typical globular proteins. This property and the reported cleavage of light chains into halves by proteolytic enzymes (Solomon and McLaughlin, 1969; Karlsson *et al.*, 1969), which was confirmed in this work for rabbit light chains, support the "domain" hypothesis, proposed by Edelman *et al.* (1969). An interesting finding, finally, is the fact that the light-chain monomer has a conformation, as measured by optical rotatory dispersion and circular dichroism, slightly different from that of the dimer.

Materials and Methods

Preparation of rabbit IgG and separation of its heavy and light chains was performed essentially as described in the preceding paper (Björk and Tanford, 1971a). The only modification was that the procedure was scaled up so that 1g of reduced and carboxylated IgG was applied to a 6 × 115 cm column of Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N. J.) in 1 M propionic acid. The separation between heavy and light chains on this column was equally good as, and in some instances even better than, that obtained on the smaller column used previously, and the recoveries were also comparable. The light-chain peak had completely emerged from the column after

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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).

about 48 hr, and dialysis of the solution (about 450 ml) against 6000 ml of 0.02 M sodium phosphate buffer (pH 7.0), containing 0.1 M sodium chloride, was started immediately. The dialysate was changed three times during the following 60 hr. The pH of the protein solution was checked and was found to be 7.0 after the completion of the dialysis procedure.

Separation of the renatured light chains into monomers and dimers was accomplished by chromatography on Sephadex G-75. The dialyzed solution was concentrated to 12 ml by ultrafiltration through a Diaflo UM 10 membrane (Amicon Corp., Lexington, Mass.), and was then applied to a 5 × 145 cm column of Sephadex G-75 in 0.02 M sodium phosphate buffer (pH 7.0) + 0.1 M NaCl. The elution rate of this column was 60 ml/hr, and 15-ml fractions were collected. Because the two resulting peaks were incompletely resolved, they were rechromatographed under the same conditions used for the initial separation. Finally, the protein solutions were concentrated by ultrafiltration as above and were stored frozen at −20° until used.

Immunodiffusion analyses and gel chromatography of fully reduced and carboxymethylated proteins in 6 M Gdn·HCl[2] were carried out as described in the preceeding paper (Björk and Tanford, 1971a).

Sedimentation velocity and sedimentation equilibrium experiments were performed in essentially the same manner as in the study of heavy chain (Björk and Tanford, 1971a). Since no problems with aggregation of either light-chain monomers or dimers were encountered, the cells were filled at room temperature and the centrifuge was operated at 25° instead of at 5°. Sedimentation velocity experiments were performed in 12-mm 4-deg single-sector cells, run at 56,100 or 59,780 rpm. In addition to meniscus depletion experiments (Yphantis, 1964), a few low-speed sedimentation equilibrium runs were also made. The procedure recommended by Chervenka (1969a) was followed, and the results were calculated in the conventional way, *i.e.*, the fringes were labeled by means of a separate determination of the initial concentration. The value for the partial specific volume used in the calculations of molecular weights from sedimentation equilibrium data was estimated from the amino acid composition of rabbit light chains (Crumpton and Wilkinson, 1963) and was found to be 0.73. The same value was used both for the monomer and dimer form of light chain. Furthermore, since this value is only approximate, it was also used for the effective partial specific volume in 6 M Gdn·HCl.

Diffusion coefficients at different protein concentrations were also determined in the analytical ultracentrifuge. The protein solutions were first dialyzed for 40–48 hr against 0.02 M sodium phosphate buffer (pH 7.0), containing 0.1 M NaCl. The solvent was layered on top of the solution in a 12-mm 2.5-deg double-sector, capillary synthetic boundary cell at 2000–3000 rpm, and the rotor was then slowly accelerated to 6166 rpm. The heavy AnJ rotor was used, and the temperature was regulated at 20°. The diffusion rate was followed by interference optics and exposures were taken 8 min apart. The results were calculated as described by Chervenka (1969b); a similar procedure was originally used by Schumaker and Schachman (1957) in conjunction with absorption optics. The total fringe shift across the diffusing boundary, J, was determined, and the radial positions of the $J/4$ and $3J/4$ fringe were located. The slope of a plot of the square of the distance between these two positions *vs.* time is proportional to the diffusion coefficient, the proportionality constant being 3.64, which is a constant derived from probability tables for one-fourth and three-fourths concentration levels of a Gaussian concentration distribution. This method is thus based on the assumption that the shape of the diffusing boundary is Gaussian. The product of the zero time correction and the diffusion coefficient was less than 10^{-4} cm^2 in all experiments reported; a higher value is indicative of insufficient sharpness of the initial boundary (Creeth and Pain, 1967).

Viscosity measurements were made at 25.00 ± 0.01° with Cannon-Manning semimicroviscometers, having flow times for water of 240–260 sec. Flow times for protein solutions ranged from 12 to 60 sec over the values for the solvent (which was 0.02 M sodium phosphate buffer (pH 7.0) + 0.1 M NaCl), and were reproducible to at least ±0.4 sec. A total of 8–10 individual flow time measurements were made for the solvent and for each protein concentration, and the average of these was computed. The results were plotted as the reduced viscosity, η_{sp}/c, *vs.* proteins concentration, c, in grams per cubic centimeters. The intercept of the resulting straight line at $c = 0$ is the intrinsic viscosity, $[\eta]$. The specific viscosity, η_{sp}, is equal to $(t\rho - t_0\rho_0)/t_0\rho_0$, where t is the flow time and ρ the density; subscript zero refers to measurements with solvent. The density of each protein solution was computed from the protein concentration, the partial specific volume of the protein (0.73, see above) and the density of the solvent, which was measured pycnometrically. The Huggins constant, k, was calculated from $\eta_{sp}/c = [\eta] + k[\eta]^2c$.

Optical rotatory dispersion and circular dichroism spectra were measured essentially as described in the preceeding paper (Björk and Tanford, 1971a). The spectra were recorded at 25–27°, using protein concentrations of 0.75–1.0 mg/ml and cells with from 1-cm to 0.5-mm path length. The optical density was never allowed to exceed 1.5 in the case of optical rotatory dispersion and 1.0 in the case of circular dichroism. Solvents were 0.02 M sodium phosphate buffer (pH 7.0) + 0.1 M NaCl or 0.01 M NaCl of varying pH. The value for the mean residue weight used in the calculations was taken as 108 both for nonspecific rabbit IgG and for light-chain monomer and dimer (Dorrington *et al.*, 1967).

Optical rotation at 225 mμ was also used to measure pH-transition curves. Protein solutions, having concentrations of about 0.2 mg/ml, were dialyzed against 0.01 M NaCl (pH 7.0) and pH was then adjusted to the desired value with 1 N HCl. The rotation was measured after 24–28 hr, and of selected samples also after 64 hr in order to ascertain that equilibrium had been reached. In reverse experiments pH was first adjusted to 1.4 and after 16–20 hr readjusted to higher values. Measurements were then made after another 24 hr.

Papain digestion was performed after a 24-hr dialysis of the protein solution (4 mg/ml) against 0.1 M sodium phosphate buffer (pH 7.0), containing 0.002 M EDTA. Mercaptoethanol (final concentration 0.01 M) and papain (Worthington Biochemicals, Freehold, N. J.) were added, and the solution was placed in a water bath at 37°. A papain to protein ratio of 1:100 (wt/wt) was used. The digestion was allowed to continue for 15 min or 2 hr and was stopped by cooling and by addition of iodoacetamide to a concentration of 0.015 M. The solution was then lyophilized for gel chromatography in 6 M Gdn·HCl.

All protein concentrations were determined spectrophotometrically in a Cary 15 spectrophotometer (Cary Instruments, Monrovia, Calif.). Extinction coefficients at 280 mμ for 1% (wt/v) solutions in 1-cm cells were obtained as described earlier (Björk and Tanford, 1971a), and were found to be

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

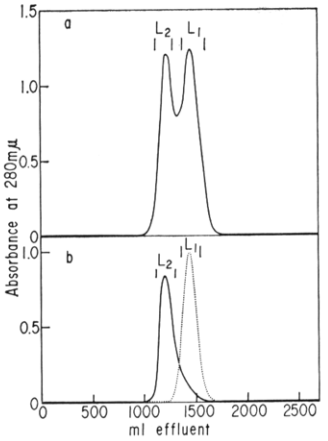

FIGURE 1: Chromatography studies. (a) Gel chromatography of renatured light chains on Sephadex G-75 in 0.02 M sodium phosphate buffer (pH 7.0), containing 0.1 M NaCl. 380 mg in 12 ml of solution was applied to a 5 × 145 cm column. (b) Rechromatography of the monomer (L_1) and dimer peaks (L_2) separately on the same column as in part a. 125 mg of dimer and 135 mg of monomer were applied to the column. The vertical lines indicate those fractions used for further analyses.

11.9 ± 0.1 (7 determinations) for the light-chain monomer and 12.4 ± 0.2 (9 determinations) for the dimer. The solvent used for these measurements was 0.01 M sodium phosphate buffer (pH 7.0).

Results

Preparation. Isolation of light chains from rabbit IgG was found to be a much easier task than isolation of heavy chains from the same source (Björk and Tanford, 1971a). No problems with aggregation were encountered and the length of exposure to the dissociating agent, 1 M propionic acid, did not seem to significantly affect the light chains (see below). Consequently, separation of light chains from heavy chains could be performed by gel chromatography on a large scale without any adverse effect on the light chains. Neither was the method for renaturation of light chains critical; dialysis against 0.02 M sodium phosphate buffer (pH 7.0), containing 0.1 M NaCl, was used routinely.

The light-chain preparation obtained in this manner was found to contain two components by sedimentation velocity experiments. These, identified below as light-chain monomers (L_1) and dimers (L_2), were separated by chromatography on Sephadex G-75 (Figure 1a). The recovery in these experiments was 90–98%. The apparent proportions between the two peaks varied somewhat with the protein concentration of the applied sample (presumably due to trailing of the dimer peak, see Figure 1b), and were also difficult to determine because of incomplete separation of the peaks. Values ranging from 55 to 65% of L_1 and from 35 to 45% of L_2 were obtained. Prolonged exposure (up to 7 days) to 1 M propionic acid before renaturation did not affect the ratio between the two peaks or their elution positions. The monomer and dimer peaks were rechromatographed individually in order to purify them further (Figure 1b). In these experiments the monomer regularly eluted as a sharp symmetrical peak, but the dimer peak always showed some trailing. This persisted even when rechromatography was performed a second time and thus does not seem to be due to a residual impurity from the monomer peak. In some experiments the two rechromato-

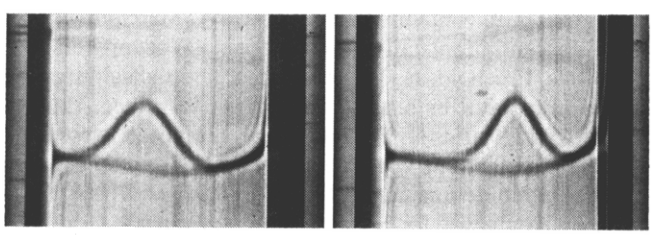

FIGURE 2: Sedimentation velocity pattern of light-chain monomer (left) and dimer (right) at 25° in 0.02 M sodium phosphate buffer (pH 7.0), containing 0.1 M NaCl. 59,780 rpm; 2.5-deg double-sector cell; protein concentration 5.0 mg/ml; base angle 35 (left) and 40° (right). Both pictures were taken 140 min after top speed was reached.

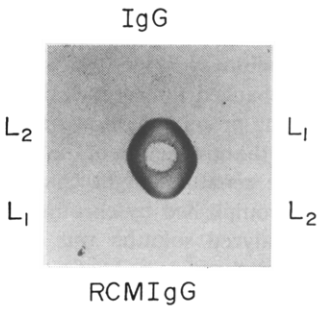

FIGURE 3: Immunodiffusion of rabbit IgG, mildly reduced and carboxymethylated IgG (RCMIgG) and light-chain monomer (L_1) and dimer (L_2) against goat antirabbit light chain. Protein concentrations were 1 mg/ml.

TABLE I: Molecular Weights of Rabbit Light-Chain Monomer and Dimer by Sedimentation Equilibrium.[a]

Concn (mg/ml)	Speed (rpm)	Mol Wt
Monomer		
0.14	35,600	23,100
0.14	39,460	22,400
0.14	42,040	22,400
0.34	42,040	22,600
0.12	44,770	23,800
2.40	15,220	24,300[b]
Dimer		
0.13	23,150	46,100
0.33	23,150	46,100
0.13	29,500	42,600
0.13	29,500	43,800
0.33	29,500	41,700
0.13	33,450	44,500
0.34	33,450	41,600
2.40	11,272	43,700
0.13 (in 6 M Gdn·HCl)	50,740	26,000

[a] All runs were meniscus depletion runs except those at 2.4-mg/ml protein concentration, which were conventional low-speed experiments. The solvent was 0.02 M sodium phosphate buffer (pH 7.0) except where noted. [b] Curved plot. The molecular weight given is that calculated from the slope at the meniscus.

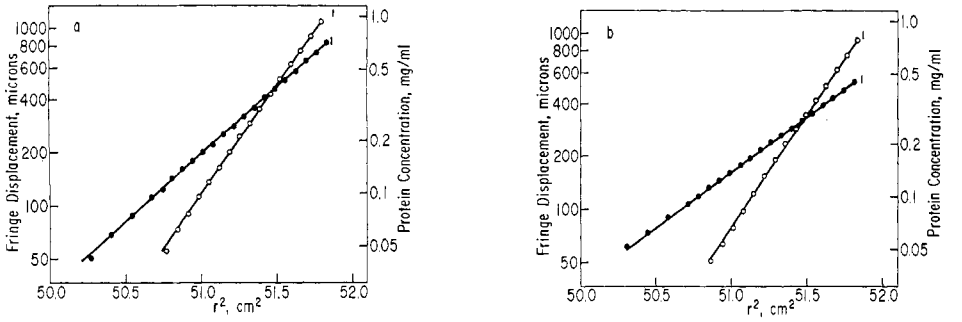

FIGURE 4: Meniscus depletion sedimentation equilibrium of light-chain monomer and dimer at 25° in 0.02 M sodium phopshate buffer (pH 7.0), containing 0.1 M NaCl. (a) Monomer; 35,600 rpm, 0.14 mg/ml (filled circles); 44770 rpm, 0.12 mg/ml (open circles). (b) Dimer; 23150 rpm, 0.13 mg/ml (filled circles); 33450 rpm, 0.13 mg/ml (open circles). The vertical lines indicate the cell bottoms.

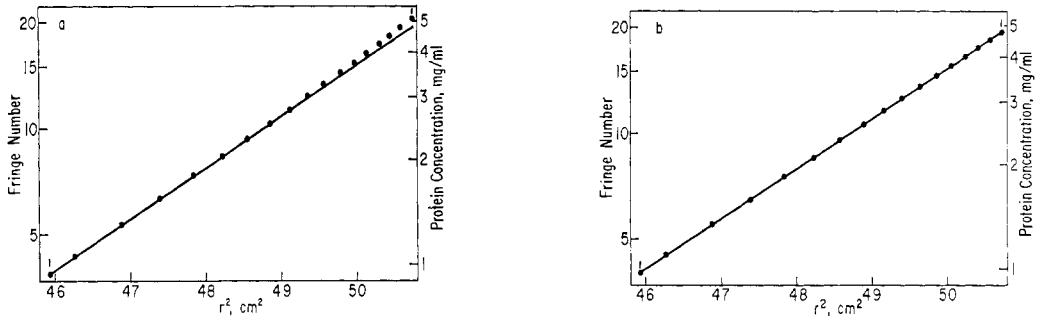

FIGURE 5: Conventional low-speed sedimentation equilibrium of light-chain monomer and dimer at 25° in 0.02 M sodium phosphate buffer (pH 7.0) +0.1 M NaCl. (a) Monomer; 15,220 rpm, 2.40 mg/ml. (b) Dimer; 11,272 rpm, 2.40 mg/ml. The vertical lines indicate the meniscus and the bottom of the cell.

graphed peaks were dialyzed against 1 M propionic acid for 24–36 hr; they were then renatured and chromatographed on Sephadex G-75 in phosphate buffer a third time. Elution patterns identical to those in Figure 1b were obtained, a further indication that neither of the two peaks is an artifact caused by the propionic acid treatment.

Purity. Both light-chain monomer and dimer were fully reduced and carboxymethylated in 6 M Gdn·HCl and then chromatographed on an agarose column in this solvent (Fish *et al.*, 1969). The elution position off this column gives an estimate of the molecular weight of a polypeptide chain, and in addition, chains of different sizes can be quantitatively separated. Both forms eluted as one peak at a position corresponding to a molecular weight of about 24,000 and no trace of material with higher or lower molecular weight was found. Moreover, in immunodiffusion experiments, neither monomer or dimer showed any precipitin line against goat antirabbit Fc. These two experiments show that the preparations were completely free of heavy chain. The extent of the separation between the monomer and dimer peaks achieved by gel chromatography was checked by sedimentation velocity experiments of the two fractions (Figure 2). In both cases symmetrical peaks were obtained, and no impurities were evident, but due to the relatively low sedimentation coefficients and rapid diffusion, up to 10% of contaminating material, such as monomer present in the dimer fraction and *vice versa*, could have escaped detection. Immunodiffusion analyses of both light-chain monomer and dimer against goat antirabbit light chain gave only one precipitin line for each form, and also showed complete immunological identity between the two (Figure 3).

Molecular Weights. The molecular weights of both light-chain peaks, obtained by chromatography on Sephadex G-75, were determined at pH 7 by sedimentation equilibrium methods (Table I). All experiments were meniscus depletion runs, except those at protein concentrations of 2.4 mg/ml, which were conventional low-speed experiments. The average molecular weights, calculated from all runs of each of the two fractions, were 23,000 and 44,000, which shows that the two peaks are composed of light-chain monomers and dimers, respectively. In this context it should again be noted that the value for $\bar{v}$, 0.73, was estimated from the amino acid composition, and hence the calculated molecular weights may only be approximate. Nevertheless, at least the monomer value agrees well with the established molecular weight of light chains (Marler *et al.*, 1964; Small and Lamm, 1966; Edelman *et al.*, 1969). The plots of the logarithm of fringe displacement or fringe number *vs.* the square of the distance from the center of rotation were linear for all meniscus depletion experiments from a fringe displacement of 50–70 μ to the bottom of the cell (Figure 4), and also throughout the cell for the low-speed run performed with light-chain dimer (Figure 5b). In the latter type of experiment, however, the monomer plot showed an upward curvature starting at a protein concentration of about 2 mg/ml (Figure 5a). The molecular weight at the meniscus was 24,300, but at the bottom of the cell about 28,000. These results show the presence of only one molecular size species in the dimer fraction at protein concentrations from about 0.1 to about 4 mg per ml and also in the monomer fraction from about 0.1 to about 2 mg per ml. At higher protein concentrations dimerization of the monomer occurs, a fact also indicated by several hydrodynamic experiments reported below.

A molecular weight of 26,000 was obtained for light-chain

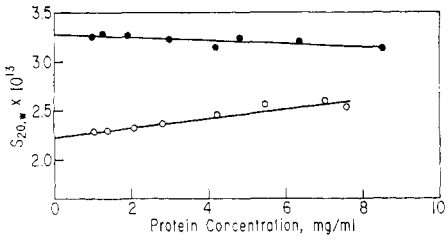

FIGURE 6: Sedimentation coefficients of light-chain monomer (open circles) and dimer (filled circles) at different protein concentrations in 0.02 M sodium phosphate buffer (pH 7.0) +0.1 M NaCl.

dimer in 6 M Gdn·HCl, pH 7.0 (Table I). In the calculation of this molecular weight the same estimated value as was used for $\bar{v}$ in dilute salt solutions, 0.73, was also used for the effective partial specific volume in 6 M Gdn·HCl. Because of preferential binding of Gdn·HCl, the latter parameter may differ from $\bar{v}$ by 0.01–0.02 (Hade and Tanford, 1967; Reisler and Eisenberg, 1969), and molecular weights in this solvent are therefore subject to a large uncertainty. In view of this, the molecular weight obtained indicates that the light chain dimer is completely dissociated in 6 M Gdn·HCl, and that thus only noncovalent bonds hold the two chains together.

Shape. Sedimentation coefficients of light-chain monomer and dimer were determined at several protein concentrations and have been plotted as a function of concentration in Figure 6. The data extrapolate to a sedimentation constant at zero protein concentration of 2.23 ± 0.03 S for the monomer and 3.29 ± 0.04 S for the dimer. The slope of the regression line is positive for the monomer, which indicates that dimerization occurs as the concentration is increased. The light chain dimer, however, shows a normal concentration dependence of its sedimentation coefficient.

Diffusion coefficients of the monomer and the dimer, measured at different protein concentrations, are shown in Figure 7. The extrapolated values for the diffusion constant at zero protein concentration are 8.9 ± 0.3 cm^2/sec for the monomer and 6.8 ± 0.2 cm^2/sec for the dimer. The uncertainty of these values is greater than that of the sedimentation constants, because diffusion experiments are more difficult to carry out properly, and also because fewer measurements were made. The diffusion coefficient of the monomer shows a negative concentration dependence, which is in accord with sedimentation velocity and low-speed sedimentation equilibrium results, whereas the diffusion coefficient of the dimer is virtually independent of protein concentration.

Frictional ratios, f/f_{min}, were calculated both from sedimentation and from diffusion constants, in both cases together with average values for the molecular weight, measured by

TABLE II: Some Physicochemical Parameters of Rabbit Light-Chain Monomer and Dimer.

	Monomer	Dimer
$s^0_{20,w}$ (sec)	2.23×10^{-13}	3.29×10^{-13}
$D^0_{20,w}$ (cm^2/sec)	8.9×10^{-7}	6.8×10^{-7}
$[\eta]$	4.3	4.8
Huggins constant, k	2.1	1.2
Molecular weight		
From sedimentation equilibrium	23,000[a]	44,000[a]
From s, D	22,800	44,100
From $s^0_{20,w}$ and $[\eta]$	24,100	45,800
f/f_{min}		
From $s^0_{20,w}$	1.30	1.35
From $D^0_{20,w}$	1.28	1.34

[a] Average of all values in Table I.

sedimentation equilibrium (Table II), and with the estimated value of $\bar{v}$. The frictional ratio gives an indication of the shape and hydration of the protein molecule, values from 1.1–1.25 being typical for sparsely hydrated globular proteins (Tanford, 1961). The values for light-chain monomer and dimer are shown in Table II. The two ways of calculating the frictional ratio gave results in very close agreement. Both monomer and dimer have values for f/f_{min} higher than for typical globular proteins; moreover, the dimer has a higher value than the monomer. These data suggest that both light-chain monomer and dimer deviate somewhat from a compact globular shape or possess a larger amount of hydration than most typical proteins, and furthermore that the dimer is the more asymmetric or more hydrated of the two.

A third hydrodynamic parameter, namely the intrinsic viscosity, was also determined. Figure 8 shows the reduced viscosity, η_{sp}/c, plotted as a function of protein concentration, the intercept of this plot at zero protein concentration being the intrinsic viscosity. This was found to be 4.3 ± 0.1 cm^3/g for the light-chain monomer and 4.8 ± 0.1 cm^3/g for the dimer. These values are somewhat larger than for typical compact globular proteins, which generally have intrinsic viscosities of less than 4.0 (Tanford, 1961). These larger values are in agreement with the frictional ratios reported above. The Huggins constant, k, which is a measure of the slope of a plot such as one of those in Figure 8, was also computed. The monomer gave a value of 2.1, which is normal for globular proteins (Tanford, 1961), but the value for the dimer was only 1.2. A higher concentration dependence of the intrinsic viscosity of the monomer is consistent with the

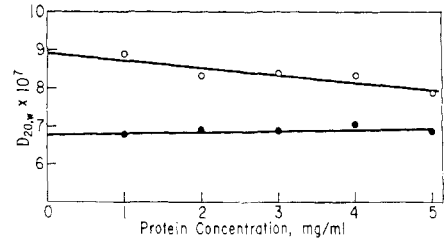

FIGURE 7: Diffusion coefficients of light-chain monomer (open circles) and dimer (filled circles) at different protein concentrations in 0.02 M phosphate buffer (pH 7.0) + 0.1 M NaCl.

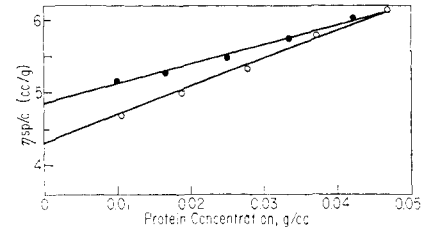

FIGURE 8: Reduced viscosity of light chain monomer (open circles) and dimer (filled circles) as a function of protein concentration. The solvent was 0.02 M sodium phosphate buffer (pH 7.0) + 0.1 M NaCl.

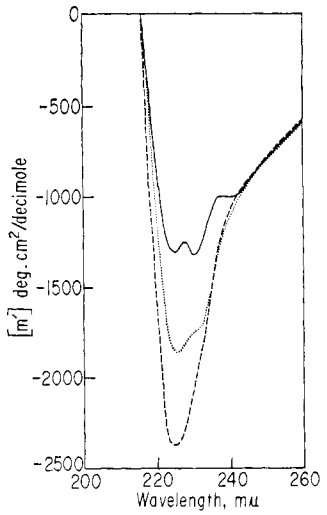

FIGURE 9: Optical rotatory dispersion spectra of rabbit IgG (solid line), light-chain monomer (broken line) and light-chain dimer (dotted line) at 25–27° in 0.01 M NaCl (pH 7.0). Protein concentrations were 0.8–1.0 mg/ml.

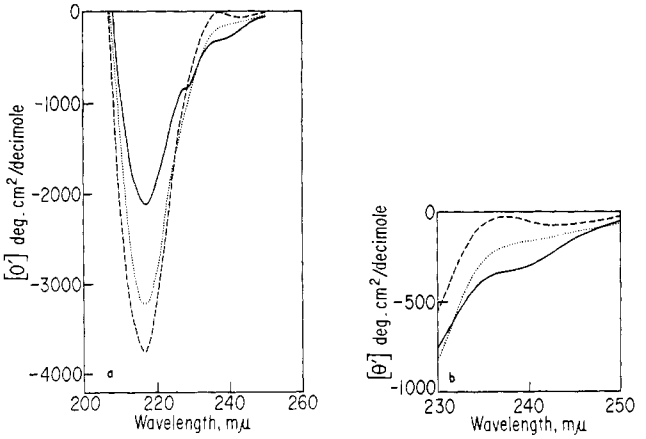

FIGURE 10: Circular dichroism spectra of rabbit IgG (solid line), light-chain monomer (broken line) and light-chain dimer (dotted line) at 25–27° in 0.01 M NaCl (pH 7.0), with protein concentrations of 0.8–1.0 mg/ml. (a) Wavelength region 208–250 mμ. (b) Wavelength region 230–250 mμ on a larger scale.

anomalous concentration dependence of its sedimentation and diffusion coefficients, but the reason for the somewhat too low Huggins constant for the dimer is not known.

In order to test for the self-consistency of all the hydrodynamic data presented, molecular weights were calculated from sedimentation and diffusion constants, using the Svedberg equation (Svedberg and Pedersen, 1940), as well as from sedimentation constants and intrinsic viscosities by use of the Scheraga–Mandelkern equation (Scheraga and Mandelkern, 1953). In these calculations the estimated value for $\bar{v}$, 0.73, was used, and β in the Scheraga–Mandelkern equation was assumed to be 2.12×10^{-6}. This is the commonly accepted value for globular proteins and was used since the deviation of either light-chain monomer or dimer from a compact globular shape cannot be very large. Furthermore, β is rather insensitive to large variations of both shape and hydration of the molecules. The resulting molecular weights are given in Table II. They clearly agree very well with those measured independently by sedimentation equilibrium.

Optical Rotatory Dispersion and Circular Dichroism Spectra. The optical rotatory dispersion spectrum of light-chain monomer and dimer is shown in Figure 9, and the well-established spectrum of IgG is also given for comparison. The two light-chain spectra are rather different from the IgG spectrum; in particular the Cotton effect at 240 mμ is missing and the rotation is more negative than that of IgG below about 240 mμ. Differences between the two light-chain spectra themselves are also obvious and well outside experimental error. The monomer shows only one trough at 225 mμ with a reduced mean residue rotation of −2400, whereas the dimer, in addition to its shallower main trough at 225 mμ with an [m'] of only −1850, also exhibits a shoulder at about 230 mμ. Both the 225 mμ and the 230-mμ minima are present also in the optical rotatory dispersion spectrum of native IgG, but have lower magnitudes in this spectrum.

The same general features are borne out also by the circular dichroism measurements (Figure 10). The circular dichroism curves of the two light-chain forms both differ from that of IgG and also from each other over the whole wavelength range studied, *i.e.*, from 250 to 210 mμ. The shoulder seen in the IgG spectrum at about 238 mμ is clearly resolved into a negative band at 242 mμ and a positive band at 237 mμ in the light-chain monomer spectrum, but is not evident in the dimer spectrum. The IgG curve furthermore has a shoulder at 228 mμ which is not seen in either light-chain spectrum. All three curves have minima at 217 mμ but with different magnitudes of their ellipticities, the reduced mean residue ellipticity of the light-chain monomer being more negative (−3800) than that of the dimer (−3200), which in turn has more negative ellipticity than native IgG (−2100).

Denaturation by pH. The pH denaturation of both light-chain monomer and dimer was followed by optical rotation at 225 mμ, and the curves presented in Figure 11 were obtained. Both forms undergo a rather limited conformational change as pH is lowered. This change starts at about pH 3.2 for the dimer, but only at about pH 2.6 for the monomer. It is complete at pH 1.4 for both forms. Optical rotatory dispersion spectra were taken at pH 1.4; they showed a single minimum at 225 mμ for both forms, and the 230-mμ shoulder in the dimer curve had disappeared. The transition curve is rather broad, reflecting the presence of many individual species of light chains in both forms, and a theoretical interpretation is therefore difficult to make. The magnitudes of the observed changes in optical rotation are rather small,

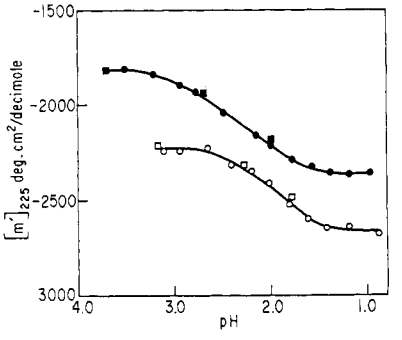

FIGURE 11: Effect of pH on optical rotation at 225 mμ, measured in 0.01 M NaCl at 25–27°, of light-chain monomer (open symbols) and dimer (filled symbols). Circles represent measurements in which pH was lowered from 7, and squares measurements in which pH was first lowered to 1.40 and then raised.

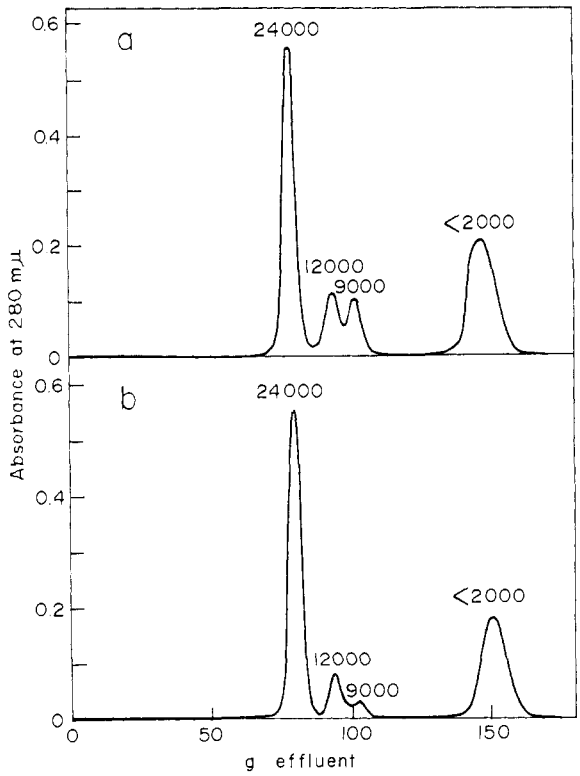

FIGURE 12: Gel chromatography of fully reduced and carboxymethylated papain digests of (a) light-chain monomer and (b) light-chain dimer on 6% agarose in 6 M Gdn·HCl. The digestions were performed for 15 min with a papain to protein ratio of 1:100 (wt/wt), and about 3.5 mg of the digest was applied to the column. The effluent emerging from the column was determined by weight instead of by volume.

especially when compared to similar pH–denaturation studies of a Bence-Jones protein (Jirgensons *et al.*, 1966) and human myeloma light chains (R. W. Green, personal communication). The Bence-Jones protein investigated exhibited a three- to -fourfold decrease in rotation at 224 mμ when exposed to pH 1.85 and also a shift in the optical rotatory dispersion minimum from 224 to about 210 mμ. Obviously rabbit light chains are much more resistant to pH denaturation than their human counterparts.

Attempts were also made to investigate whether the denaturation of light-chain dimer by pH is accompanied by a dissociation into monomers. Meniscus depletion sedimentation equilibrium experiments were performed in 0.01 M NaCl, pH 2.3 and 1.4, in the presence of 0.5% sucrose, at a speed of 35,600 rpm with the same protein concentration, 0.2 mg/ml, as was used for the transition curve. At pH 2.3, the approximate midpoint of the transition curve, a straight plot of the logarithm of fringe displacement *vs.* r^2, giving a molecular weight of 41,000, was obtained. This value indicates that no appreciable dissociation has taken place at pH 2.3, since a low molecular weight of undissociated dimer would be expected, due to charge effects caused by the low ionic strength and pH (Johnson *et al.*, 1954). At pH 1.4, where the transition measured by optical rotation is complete, a curved plot was obtained, with the initial slope giving a molecular weight of 29,000 and the slope at the bottom of the cell a molecular weight of about 43,000. Obviously dissociation into monomers is incomplete even at pH 1.4. Therefore it seems that the conformational change that occurs when pH is lowered preceeds the dissociation of light-chain dimers into monomers.

Proteolytic Digestion. Several proteolytic digestions of rabbit light-chain monomer and dimer were made, mainly with papain. The digestion mixtures were analyzed after complete reduction and alkylation by gel chromatography on 6% agarose in 6 M Gdn·HCl. The elution patterns in Figure 12 were obtained. The number over each peak indicates its molecular weight, which was determined in separate experiments, in which markers were included. A large amount of both monomer and dimer remained undigested and a considerable part of both was completely digested to small peptides, indicating frequent nonspecific cleavage of the chains. The last peak does not completely consist of low molecular weight peptides, however, since some ultraviolet-absorbing impurities, probably from the mercaptoethanol, also elutes in this faction. In addition to these two peaks, two fractions with intermediate molecular weights, about 12,000 and 9000, were also found. The larger one of these has the molecular weight expected for half-light chain, and its presence thus indicates that limited proteolytic cleavage has occurred in the middle of the chain, possibly between the variable and constant regions. This has earlier been shown to be the case for Bence-Jones proteins (Solomon and McLaughlin, 1969) and for normal human light chains (Karlsson *et al.*, 1969). The existence of another gel chromatographic fraction with a lower molecular weight suggests that there is an additional specific enzyme-susceptible site somewhere in the light chain. Digestion of the dimer form consistently produced smaller amounts of the two intermediate fragments than obtained by similar treatment of the monomer, and the susceptibility of the two forms to proteolytic digestion may therefore be slightly different. Prolonged incubation, up to 2 hr, of both monomer and dimer resulted in an increased amount of low molecular weight peptides, but the yield of the two fractions with intermediate molecular weights remained virtually unchanged.

Discussion

One of the main results of the investigations reported in this paper is that free light chains, isolated from rabbit IgG by gel chromatography in 1 M propionic acid and subsequently renatured, exist in both monomeric and dimeric form at neutral pH. This was originally suggested as a possibility by Fleischman *et al.* (1962), but on the basis of very limited evidence. An interesting aspect is that the monomer and dimer forms are not in equilibrium with each other, but rather behave as apparently stable entities. They can be separated from each other by gel chromatography, and each form can be stored for long periods of time without any measurable amount of the other form appearing. The existence of monomeric and dimeric forms does not appear to be an artifact, caused by treatment with dissociating agent, 1 M propionic acid, in view of the results of several experiments, which demonstrate that this solvent does not affect the distribution between the two forms or their elution positions in gel chromatography. Our conclusion is therefore that some of the many species of light chains present in nonspecific rabbit IgG form monomers at neutral pH, when separated from their heavy-chain partners, whereas other species form dimers. The existence of stable monomers and dimers has also been shown in the case of free normal human light chains, isolated from urine (Berggård and Peterson, 1969). Moreover, some Bence-Jones proteins (*i.e.*, homogeneous,

monoclonal light chains, excreted in the urine by patients suffering from multiple myeloma) have been shown to exist as monomers, whereas others from dimers (Bernier and Putnam, 1964; Gally and Edelman, 1964). In contrast, human light chains, isolated from nonspecific IgG, seem to predominantly form dimers at neutral pH (Edelman and Gally, 1962). The underlying structural reasons for these different behaviors are not known.

The occurrence of apparently stable monomeric and dimeric forms of rabbit light chains does not preclude, however, that both forms may participate in reversible monomer–dimer equilibria, with the equilibrium constant highly favoring the monomer form for certain light-chain species and the dimer form for other species. This situation was clearly demonstrated in the monomer case. A sedimentation equilibrium experiment at high protein concentrations and several hydrodynamic measurements showed that a detectable amount of dimers form when the protein concentration is increased over 2–3 mg/ml. This dimerization must be reversible, since many sedimentation equilibrium experiments at low protein concentrations, which did not show the presence of any dimer, were performed with solutions obtained by dilution of a concentrated stock solution. No attempt to determine the association constant from these data was made, since only one sedimentation equilibrium experiment at sufficiently high concentration was performed.

A corresponding detectable dissociation of the light-chain dimer fraction into monomers at low protein concentrations is more uncertain. Two facts are consistent with a possible dissociation upon dilution, namely trailing of the dimer peak in gel chromatography (which, however, also could be due to adsorption of the protein to the gel matrix) and also the somewhat too low molecular weights obtained in most sedimentation equilibrium experiments, *i.e.*, 42,000–44,000 instead of the expected value, 46,000. The same molecular weight, however, was obtained from protein concentrations of about 0.1 to about 4 mg per ml, and no concentration dependence is thus evident. Therefore the low molecular weight of the dimer does not seem to be due to a dimer-monomer equilibrium detectable even at the lowest protein concentration studied. Such an equilibrium may exist, but with a dissociation constant so low that an appreciable proportion of monomers appears only at much lower protein concentrations. A very low dissociation constant is also indicated by the fact that the dimers are very resistant to dissociation by acid pH. A dimer of a Bence-Jones protein has actually been reported to dissociate at low protein concentrations (Bernier and Putnam, 1964), whereas free human noncovalent-linked light-chain dimers did not show any evidence of dissociation down to a concentration of 0.3 mg/ml (Berggård and Peterson, 1969).

A plausible explanation for the slightly too low dimer molecular weight would be the presence of a stable low molecular weight impurity. This does not seem to be the case, since meniscus depletion experiments at high speeds and high protein concentrations did not show any initial curvature of the plots of the logarithm of fringe displacement *vs.* r^2, which they should have done if low molecular weight material had been present. In addition, sedimentation velocity experiments did not reveal any component of smaller size. A possible reason may be that the partial specific volume of the dimer is higher than that of the monomer; the use of the same value for both, as was actually done, would lead to a too low dimer molecular weight. Finally, of course, the deviation is sufficiently small, that experimental errors in the measurements cannot be ruled out.

All hydrodynamic parameters determined show that both forms of light chains deviate somewhat from a compact globular shape, although not as much as native IgG or free heavy chain, and that the dimer is more asymmetric or possibly, but not as likely, more hydrated than the monomer. Comparative data in the literature on hydrodynamic properties of light chains are sparse; for example, no published viscosity measurements have been found. Monomers and dimers of Bence-Jones proteins generally have been reported to have higher sedimentation constants and lower frictional ratios than those we have obtained for rabbit light chains (Putnam and Stelos, 1953; Deutsch, 1955; Putnam, 1957; Bernier and Putnam, 1964), but the data have not always been extrapolated to zero protein concentration. In a recent paper by Berggård and Peterson (1969) some hydrodynamic parameters of free human light-chain monomers and dimers, isolated from urine, were determined. The sedimentation constants published for these proteins are also higher and the frictional ratios lower than for rabbit light chains; the diffusion constants are comparable, but the values obtained by Berggård and Peterson may not be exact, since they were either calculated from a Stokes radius, determined by gel chromatography, or were measured directly but not extrapolated to zero protein concentration. These comparisons suggest that free rabbit light-chain monomers and dimers may have a somewhat more extended shape than the corresponding human light-chain forms.

Recently, Edelman *et al.* (1969) have suggested that both chains of immunoglobulins consist of a series of globular regions, "domains," which are linked together by more flexible segments of the chains. Two such globular regions were proposed to exist in light chain. Our results give some support to this hypothesis. The extended shape of free rabbit light chains, indicated by the frictional ratio and the intrinsic viscosity, is certainly in agreement with such a structure. The existence of an exposed region in the middle of the light chain, a feature the domain theory also predicts, is demonstrated by the fact that proteolytic digestion can occur specifically at this point in both monomer and dimer. This has earlier been reported for human Bence-Jones proteins and normal light chains (Solomon and McLaughlin, 1969; Karlsson *et al.*, 1969), and we have extended these observations to nonspecific rabbit light chains. Karlsson *et al.* (1969) have actually shown that the two halves of human light chains, obtained by proteolytic digestion, have lower frictional ratios than the undigested chain, in strong support of the domain theory. It should be pointed out, however, that these results reflect properties of free light chains and therefore may not be strictly applicable to the structure of light chain, when it is associated with heavy chain in IgG.

From the optical rotatory dispersion and circular dichroism spectra of the two light-chain forms and the corresponding spectra of heavy chain, published in the preceding paper (Björk and Tanford, 1971a) it is obvious that some conformational change has occurred as a result of chain separation. The reason for this is that, for both the optical rotatory dispersion and circular dichroism measurements, the weighted average of the heavy-chain spectrum and the spectrum of either of the two light-chain forms clearly is not equal to the spectrum of native IgG (Dorrington *et al.*, 1967; Björk and Tanford, 1971a,b), which would have been the case, had no conformational change occurred. The distinctive features of the IgG spectrum, however, are present in either the heavy-chain spectrum or one of the light-chain spectra. The 225-mμ

trough in the optical rotatory dispersion curve of native IgG may arise mainly from light chain, since this minimum is predominant in the optical rotatory dispersion spectra of both light-chain forms. Similarly, the trough at 230 mμ and the 240-mμ Cotton effect are probably given by heavy chain (Björk and Tanford, 1971a). In circular dichroism, it may be possible that the shoulder at 238 mμ in the spectrum of IgG arises from light chain, since a negative and a positive band clearly are present in this region in the light-chain monomer spectrum. It is, however, impossible to assign the main circular dichroism trough in a similar way, since IgG, free heavy-chain, and both light-chain monomer and dimer all have minima at 217 mμ.

An interesting result is that the light-chain monomer and dimer forms have somewhat different optical properties. The optical rotatory dispersion spectrum of the dimer, for example, is less negative than the monomer spectrum over part of the wavelength range studied, and also has a shoulder at 230 mμ. The main circular dichroism minimum of the dimer at 217 mμ is also less negative than that of the monomer, and furthermore, the circular dichroism spectra in the 230–250-mμ region are clearly different. In this context it should be noted that both native and recombined IgG has more shallow optical rotatory dispersion and circular dichroism spectra than their component heavy and light chains (Dorrington *et al.*, 1967; Björk and Tanford, 1971a,b). It may therefore be that association of two IgG chains, whether like, as in light-chain dimer, or unlike, as in IgG, gives less negative magnitudes of the main circular dichroism or optical rotatory dispersion troughs of the resulting molecules. The distinctive spectra of light-chain dimer may thus be a consequence of a conformational change accompanying dimerization. On the other hand, since the existence as monomer or dimer most probably is an intrinsic property of different species of light chains, it is also possible that the species that form dimers have a certain conformation, giving specific optical rotatory dispersion and circular dichroism spectra, and as a result of this conformation are able to form dimers. The monomer species may possess a slightly different conformation, which will produce its characteristic spectra, and this conformation may allow the molecules to exist only as monomers. It is interesting that this conformational difference between monomer and dimer, which is observed by optical methods, does not seem to involve any major antigenic determinants, since the two forms showed complete immunological identity in immunodiffusion experiments.

Studies of antigen-binding activity of light chains, isolated from specific antibodies, have usually indicated that free light chains have no detectable affinity for the antigen (Utsumi and Karush, 1964; Haber and Richards, 1966; Porter and Weir, 1966); in one more recent study, however, a very low affinity was reported (Yoo *et al.*, 1967). This problem is currently under reinvestigation in our laboratory, using separated light-chain monomers and dimers. Considerations similar to those discussed in conjunction with the antigen-binding ability of heavy chain in the preceeding paper would indicate that interaction between two light chains in a dimer molecule might possibly suffice to form a weak binding site, whereas a monomeric light chain alone may not be capable of binding the antigen.

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