Talbot, D. A. (1968), Ph.D. Thesis, Dartmouth College, Hanover, N. H.
von Hippel, P. H., and Schleich, T. (1969a), *in* Structure and Stability of Biological Macromolecules, Timasheff, S. N., and Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, p 417.
von Hippel, P. H., and Schleich, T. (1969b), *Accounts Chem. Res. 2*, 257.
von Hippel, P. H., and Wong, K.-Y. (1964), *Science 145*, 577.
von Hippel, P. H., and Wong, K.-Y. (1965), *J. Biol. Chem. 240*, 3909.

Circular Dichroism Studies on the Acid Denaturation of γ-Immunoglobulin G and Its Fragments*

Etsushiro Doi and Bruno Jirgensons

ABSTRACT: Circular dichroism of human immunoglobulin G and its tryptic digestion fragments Fab(t) and Fc(t) was studied in neutral and acid solutions. In neutral solutions, all the proteins examined showed at least two circular dichroism bands in the far ultraviolet zone: a negative band centered at 217 mμ and a positive band at approximately 202 mμ. The former indicates the presence of the β structure. In hydrochloric acid (pH 2.2), the positive band at 202 mμ was shifted to a negative band at 200 mμ, whereas the negative band at 217 mμ was affected very little. At low pH, all the other bands (at 230 to 300 mμ) were strongly reduced. The negative ellipticity values at 200 mμ in acid (pH 2.2) were different for each protein but the general features of the circular dichroism curves, including those of more or less completely renatured proteins, were similar. Lower ionic strength favored disorganization and separation of the polypeptide chains in acid solution, as confirmed by sedimentation and viscosity data.

The results led us to conclude that disorganization by acid and separation of the chains were caused by electrostatic repulsive forces which overcame other interactions, such as hydrophobic and hydrogen bonds. Also, it was concluded that the disorganization in acid was incomplete and that the circular dichroism spectra were determined to a large extent by unspecified constrained polypeptide backbone conformations and amino acid side chains.

The optical rotatory dispersion of immunoglobulin G has been studied in several laboratories. Analyses by Drude and Moffitt methods have been interpreted to indicate little or no α helix in IgG[1] (Jirgensons, 1958, 1961; Winkler and Doty, 1961; Imahori and Momoi, 1962; Hamaguchi and Migita, 1964; Gould *et al.*, 1964). Several investigators have suggested that some β structure may be present in native γ-globulin (Callaghan and Martin, 1963; Imahori, 1963). Cotton effects of normal human IgG in the far ultraviolet led to the conclusion that some β structure was present (Jirgensons, 1965, 1966a,b, 1969).

Analysis of the circular dichroism of rabbit IgG by Sarkar and Doty (1966) showed a negative band near 217 mμ. The position of this CD band corresponds to that found for poly-L-lysine, poly-L-serine, and silk fibroin in the β conformation (Sarkar and Doty, 1966; Townend *et al.*, 1966; Iizuka and Yang, 1966; Quadrifoglio and Urry, 1968; Li and Spector, 1969). The same observations have been reported recently for human IgG and Bence-Jones proteins (Ross and Jirgensons, 1968; Ikeda *et al.*, 1968) and for rabbit IgG and its papain fragments (Cathou *et al.*, 1968). The positive band in the deep ultraviolet at 200–202 mμ was first reported by Ross and Jirgensons (1968). The gross conformation of papain fragments Fab and Fc has been described by Noelken *et al.* (1965). They concluded that the fragments existed in the parent molecule in the same conformation as when isolated from it.

The present work offers data on the circular dichroism spectra of human IgG and its tryptic digestion fragments, Fab(t)[1] and Fc(t), in the native state and acid-denatured state. The changes in the circular dichroism spectra of the Fc(t) fragment were studied in detail and the results were compared with those of the Fab(t) fragment, parent IgG, and myeloma IgG.

Materials and Methods

Ultra Pure guanidine hydrochloride was obtained from Mann Research Laboratories (New York, N. Y.) and was used without further purification. Iodoacetamide (Mann Re-

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[1] The abbreviations used are: IgG, Fab, and Fc, as recommended by the World Health Organization (1964); Fab(t) and Fc(t) are used for the tryptic fragments similar to the papain digestion fragments Fab and Fc; Gu·HCl, guanidine hydrochloride.

search Laboratories) was recrystallized three times from chloroform.

Preparation of Purified Normal Human IgG and Myeloma IgG. Normal human IgG was purified from commercial human IgG (fraction II, Pentex Inc., Kankakee, Ill.) by the modified procedure of Baumstark *et al.* (1964). DEAE-Sephadex A-50 was equilibrated with 0.01 M sodium phosphate (pH 6.5) and poured into a 2.5 × 40 cm column. About 40 ml of a 5% solution of IgG was loaded on the column and eluted with the same buffer. Under these conditions the IgG is not adsorbed but all other proteins are held on the column. Myeloma IgG was the same sample reported already (Ross and Jirgensons, 1968). Partial reduction and alkylation of IgG were carried out according to Edelman *et al.* (1968).

In the preliminary experiment amounts of free sulfhydryl groups in the reduced IgG molecule were determined by spectrophotometric titration with *p*-mercuribenzoate (Boyer, 1954) and colorimetric measurement with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959) in the presence of 1% sodium dodecyl sufate (Diez *et al.*, 1964). Excess reducing agent present in the protein solution was removed either by precipitation of the protein with an ethanol–1.0 M HCl (39:1) mixture at −5° (White, 1960) or by passing the protein solution through a Sephadex G-50 column (0.9 × 25 cm) which had been equilibrated with nitrogen-flushed 0.01 M sodium phosphate (pH 6.0).

Preparation of Tryptic Fragments. The method is the modification described by Edelman *et al.* (1968). Partially reduced and alkylated normal human IgG (2.0 g) was dissolved in 200 ml of 0.15 M NaCl containing 0.05 M $CaCl_2$. The solution was kept at pH 8.0 by the addition of 0.1 N NaOH at 37°. TPCK-trypsin (20 mg), which had been prepared from commercial trypsin (twice crystallized, salt free, Worthington Biochemical Corp Freehold, N. J.) by the method of Carpenter (1967), was added to the IgG solution, and the pH was maintained at 8.0 by the addition of 0.1 N NaOH at 5-min intervals at 37°. After 20 min, 40 mg of soybean trypsin inhibitor (Worthington Biochemical Corp.) was added. The sample was dialyzed against 0.02 M sodium phosphate (pH 8.0) at 4° and then centrifuged. The precipitate was washed several times with 0.3 M sodium phosphate (pH 8.0) and the washings were combined with the effluent of the chromatography described later. The supernatant solution was applied on a column of DEAE-Sephadex A-50 (3 × 50 cm) equilibrated with 0.02 M sodium phosphate (pH 8.0). Fab(t) and undigested IgG were not retarded by the column, and Fc(t) was eluted by 0.3 M sodium phosphate (pH 8.0). Fractions containing Fab(t) and undigested IgG were combined and then separated from each other by chromatography on Sephadex G-100. Fractions containing Fc(t) were combined with the supernatant solution described above and dialyzed against 0.02 M sodium phosphate (pH 8.0). The dialysate was applied on a column of DEAE-Sephadex A-50 (3 × 50 cm) equilibrated with the same buffer. A linear gradient from 750 ml of the initial buffer to 750 ml of 0.3 M sodium phosphate (pH 8.0) was applied for elution of Fc(t). Fractions containing the main component were combined and purified further by chromatography on Sephadex G-100 (3 × 90 cm) equilibrated with 0.1 M sodium phosphate (pH 8.0).

Sedimentation Analysis. All determinations were made with a Beckman–Spinco Model E ultracentrifuge at a speed of 59,780 rpm and 20.0°.

Viscosity. Viscosities were determined in a Cannon–Ubelhode dilution viscometer at 28.0°. The data were plotted as η_{sp}/C against C, where η_{sp} is the specific viscosity and C is the concentration in g/100 ml. The intrinsic kinematic viscosity $[\eta]$ was obtained by extrapolation to infinite dilution.

Molecular Weight. Molecular weights were calculated from the intrinsic viscosities and sedimentation coefficients by using the equation of Scheraga and Mandelkern (1953), in which β was assumed to be 2.16×10^6 (Schachman, 1957) and the partial specific volume of IgG and its fragments was assumed to be 0.73 cc/g (Edelman *et al.*, 1968).

Protein Concentration. Protein concentrations were determined from light absorption at 280 mμ. Extinction coefficients for a 1% solution and a 1-cm path length were assumed to be 14 for normal and myeloma IgG (Ross and Jirgensons, 1968) and the value of 15 was determined for the present preparations of Fab(t) and Fc(t).

Circular Dichroism Measurement. The new Model CD-SP Durrum-Jasco highly sensitive dichrograph was used. The most sensitive scale setting of 1×10^{-4} dichroic optical density difference (ΔE) per 1 cm on the recorder chart was used. This amounts to a sensitivity of 1×10^{-5}, but the reproducibility of the circular dichroism recordings was poorer with highly absorbing solutions, *e.g.*, in the deep ultraviolet below 195 mμ. The optical path in the near ultraviolet was 1.0 cm and it was 1.0, 0.1, or 0.05 cm in the far ultraviolet spectral region from 250 mμ down to about 185 mμ. In the measurement of the rate of disorganization, the wavelength was set at 202 mμ, and the change of ΔE was recorded as a function of time. The performance of the instrument was checked by the use of a solution of camphorsulfonic acid. All recordings were made at an ambient room temperature of 23–25°. The data were calculated as mean residue molar ellipticity, $[\theta]$. The mean residue weight of 109 was used for all proteins (Ross and Jirgensons, 1968). Reduced mean residue ellipticities, $[\theta']$, in thedilute salt solution and acid solution were calculated taking the refractive indices of water (Fasman, 1963). Since the refractive index dispersion of 6 M Gu·HCl solution is unknown, it was assumed that the ratio $[3/(n^2 + 2)]_{Gu\cdot HCl}/[3/(n^2 + 2)]_{H_2O}$ was constant at any wavelength. From the data of Kielley and Harrington (1960) the ratio was found to be 0.933 at 589 mμ. Thus, the reduced mean residue ellipticities in 6 M Gu·HCl solution were given by the following equation:

$$[\theta'] = [\theta] \times [3/(n^2 + 2)]_{H_2O} \times 0.933$$

pH Measurement. pH measurements were carried out on a Corning Model 12 research pH meter equipped with a semimicro combination electrode (cat. no. 476050). The meter readings were calibrated by 0.05 M potassium biphthalate prepared from NBS Standard Reference Material (National Bureau of Standards Office of Standards Reference Materials, Washington, D. C.) and (pH 7.00) buffer solution (Certified by Fisher Scientific Co., Pittsburg, Pa.).

Results

Characterization of Fc(t) Fragment. Preliminary experiments showed that partial reduction of normal human IgG yielded

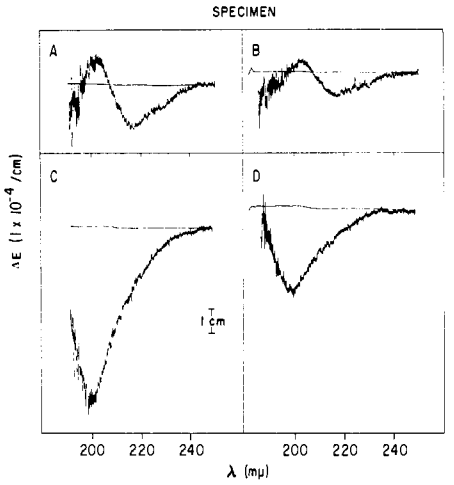

FIGURE 1: Far ultraviolet circular dichroism spectra of the Fc(t) fragment copied directly from the recorder chart paper. Curves A and B: Fc(t) in water (pH 6.3); curves C and D, Fc(t) in dilute HCl, pH 2.2. The concentrations of Fc(t) were 0.42 mg/ml for A and C, and 0.21 mg/ml for B and D. The optical path length was 0.05 cm.

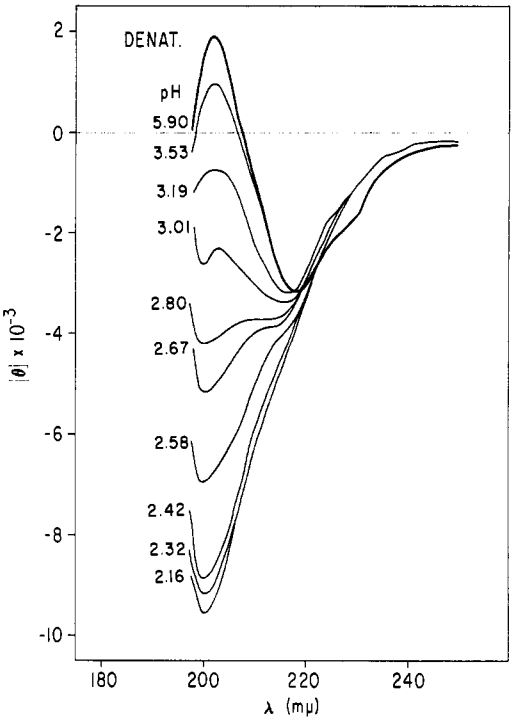

FIGURE 2: The change in the far ultraviolet circular dichroism spectrum of Fc(t) fragment during acid denaturation. A 1-cm path-length cuvet which contained 3 ml of Fc(t) solution (0.0202 mg/ml) in 0.02 M NaCl was used. Small portions of 0.1 N HCl were added with stirring. After each addition of HCl, the circular dichroism spectra and the pH of the solutions were measured. The circular dichroism measurements were made approximately 30 min after addition of acid.

8 or 9 equiv of SH groups/mole of protein when these were determined by spectrophotometric titration with *p*-mercuribenzoate or by colorimetry with 5,5′-dithiobis(2-nitrobenzoic acid). Thus, the Fab(t) and Fc(t) fragments obtained from this partially reduced and alkylated IgG contained no intermolecular disulfide bridges.

About 8 moles of base/mole of protein was consumed during the course of tryptic digestion of partially reduced and alkylated IgG for 20 min at 37°. The linear gradient elution pattern from DEAE-Sephadex A-50 indicated the presence of faster and slower eluted components other than the main component which proved to be 3.7 S. The faster eluted component showed 5.2 S by sedimentation analysis. This component disappeared under prolonged digestion (60 min), and probably was an intermediate compound.

The main component obtained by the above procedure contained only threonine as the N-terminal amino acid. Ultracentrifugal analysis showed a single component ($s_{20,w}$ = 3.7 S). Immunoelectrophoresis, using goat antiserum against the Fc(t) fragment showed a single precipitin band. The products obtained from a prolonged digestion period (60 min) or a higher substrate concentration (5%) showed broad elution patterns with slower eluting components in linear gradient chromatography on DEAE-Sephadex A-50. The main component obtained from these fractions still showed a single component in sedimentation after purification with Sephadex G-100 but contained several N-terminal amino acids other than threonine. The Fc(t) fragment used throughout the following experiments was obtained by the standard set of conditions for digestion: a substrate concentration of 1% and a digestion period of 20 min at 37°, pH 8.0, and the enzyme to substrate ratio 1:100.

Far Ultraviolet Circular Dichroism Spectra of the Fc(t) Fragment. The reproducibility and reliability of the circular dichroism instrument in the far ultraviolet region is shown in Figure 1, using two different concentrations of the Fc(t) fragment in water and in HCl in pH 2.2. A negative band at 217 mμ characteristic of β structure, and a positive band at 202 mμ, were observed in neutral solution.

A single negative band at 200 mμ was observed in acid solution. Although the wavelength of the negative band was not the same as that of the typical random coil poly-α-amino acids, it is indicative of disorganized conformation.

Circular Dichroism Spectral Titration of Fc(t) Fragment at Acidic pH. Small portions of 0.1 N HCl were added with stirring to a 1-cm path-length cuvet which contained 3 ml of an Fc(t) solution in 0.02 M sodium chloride and a magnetic stirring bar. After each addition of HCl, the circular dichroism spectrum and the pH of the solution were measured. Reverse titration was pursued in the same way using 0.096 N NaOH. Several intermediate curves obtained during forward titration are shown in Figure 2. It was shown that the positive peak at 202 mμ observed in the native protein shifted to a negative peak at 200 mμ with increasing concentration of acid, whereas the negative peak at 217 mμ was affected relatively little and was still observed as a shoulder on the curves at acidic pH. The circular dichroism spectra of the renatured protein were similar to those of the native protein. A large change in $[\theta]_{202}$ was observed at the inflection point near pH 2.7 (forward titration) and near pH 3.0 (reverse titration) but not much change was found at 217 mμ. The small increase in the negative ellipticity at 217 can be explained as the effect of the large negative band at 200 mμ.

Denaturation of Fc(t) Fragment with 6 M Guanidine Hy-

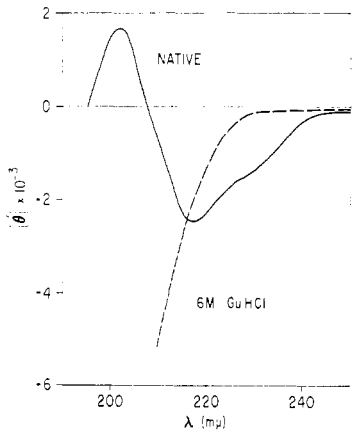

FIGURE 3: The far ultraviolet circular dichroism spectra of Fc(t) denatured with 6 M Gu·HCl for 2 hr at room temperature. Fc(t) was dissolved in 6 M Gu·HCl containing 0.01 M Na-phosphate of pH 7.0. The protein concentration was 0.22 mg/ml and the optical path length was 0.05 cm.

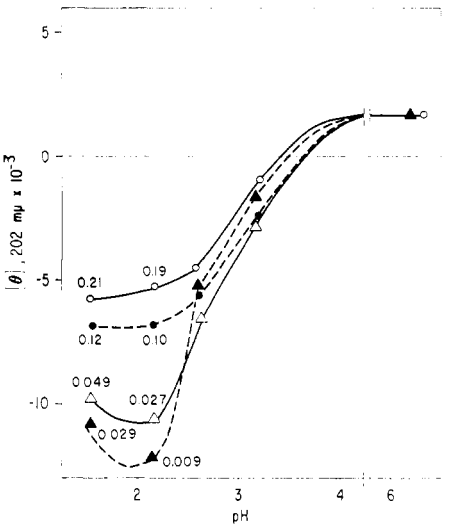

FIGURE 4: The effect of ionic strength on the acid denaturation of Fc(t). The protein was dissolved in water and 0.02 M, 0.1 M, and 0.2 M NaCl, respectively, were added. The initial protein concentration was 0.342 mg/ml. Solutions of 0.1 N and 1.0 N HCl then were added to 1 ml of the solutions to get the desired pH. After 40–60 min, the circular dichroism spectra were recorded. The optical path was 0.05 cm. Since the initial ionic strengths were changed by addition of acid, the corrected ionic strengths are indicated at each curve.

drochloride. In Figure 3 is shown the circular dichroism spectrum of Fc(t) fragment denatured with 6 M Gu·HCl. Although the position of the negative band could not be determined because of excessive absorption by the solvent, the change of negative ellipticity near 217 mμ indicates reduction of β structure in this solvent. No more circular dichroism change was observed of these solutions after either acidification to pH 2.2 or heating for 2 hr at 50°.

Acid Denaturation of Fc(t) Fragment as a Function of Time. The time course of disorganization of the Fc(t) fragment by acid was recorded continuously with the change in ellipticity at 202 mμ, and it was found that the major change occurred immediately after addition of the acid and was followed by a gradual increase in negative ellipticity. The renaturation process which was initiated by the addition of alkali occurred in two steps. The complete recovery of ellipticity at 202 mμ to the original positive value took approximately 70 hr at room temperature. Renaturation of the Fc(t) molecule seemed to be affected by several factors, such as protein concentration, final pH, and the mixing procedure during the addition of alkali.

Effect of Ionic Strength on the Acid Denaturation of Fc(t). Since it has been shown that a higher concentration of salt caused aggregation of IgG at low pH (Phelps and Cann, 1957), the effect of ionic strength on the acid disorganization of the Fc(t) fragment was investigated (Figure 4). Fc(t) was dissolved in water and in NaCl of various concentrations and then acidified by the addition of HCl. Since the ionic strengths of the acidic solutions were different from the initial values because of the addition of acid, the corrected values are indicated in Figure 4. The circular dichroism spectra of the Fc(t) fragment at neutral pH were not affected by the change in ionic strength. However, a higher concentration of salt inhibited the disorganization by acid, as indicated by the change of the ellipticity at 202 mμ. The curves at low ionic strength show a minimum near pH 2.0. It can be assumed that the high concentration of the chloride ions at a pH lower than 2.0 counteracted the effect of acid. Maximum disorganization was observed in 0.01 N HCl solution.

Comparison of Circular Dichroism Spectra of Normal IgG, Myeloma IgG, and Fab(t) and Fc(t) Fragments. The circular dichroism spectra of IgG and fragment Fab(t) in neutral and acid solutions are shown in Figures 5–8, and the ellipticities are listed in Table I. The negative ellipticities at 217 mμ in neutral solutions are of nearly the same magnitude, whereas the ellipticities of the positive band at 202 mμ are different for each protein. Fragment Fab(t) showed the highest value (Figure 8). Reduction and alkylation of the interchain disulfide bonds did not cause any significant change in the circular dichroism spectra of normal IgG and myeloma IgG in neutral solution. When the interchain disulfide bonds were intact, they hampered the disorganization by acid (Figure

TABLE I: Reduced Mean Residue Ellipticities[a] of IgG and Its Fragments at Neutral and Acidic pH.

Protein	Neutral pH		pH 2.2	
	$[\theta']_{217}$	$[\theta']_{202}$	$[\theta']_{217}$	$[\theta']_{200}$
Normal IgG	−2500	2600	−3500	−4300
Partially reduced and alkylated normal IgG	−2800	3000	−3500	−5900
Myeloma IgG	−2700	1200	−3000	−2500
Partially reduced and alkylated myeloma IgG	−3000	1700	−3000	−5900
Fab(t)	−3000	4700	−3500	−5800
Fc(t)	−2500	1900	−3600	−8000

[a] Expressed in degrees decimole^{-1} cm^2.

TABLE II: Sedimentation Coefficients, Intrinsic Viscosities, and Molecular Weights of IgG and Its Fragments at Neutral and Acidic pH.

Protein	Solvent (M)	pH	$s^0_{20,w}$ (S)	$[\eta]^a$	Mol Wt^b
Partially reduced and alkylated normal IgG	NaCl (0.1)–Na-phosphate (0.01)	7.0	6.70	0.083	170,000
Partially reduced and alkylated normal IgG	HCl (0.01)	2.2	2.42^c	0.210	58,000
Fab(t) fragment	NaCl (0.1)–Na-Phosphate (0.01)	7.0	3.64	0.048	51,000
Fab(t) fragment	HCl (0.01)	2.2	1.98^c	0.120	33,000
Fc(t) fragment	NaCl (0.1)–Na-phosphate (0.01)	7.0	3.61	0.050	51,000
Fc(t) fragment	HCl (0.01)	2.2	1.46	0.198	26,000
Fc(t) fragment	HCl (0.01)–NaCl (0.1)	2.2	2.3,^{d} 6.5	0.097	

^{a} Expressed in 100 ml/g. ^{b} Calculated by the equation of Scheraga and Mandelkern (1953). ^{c} The peak was skewed with fast-sedimenting components. ^{d} Two peaks of similar size were observed.

5-7). The change in negative ellipticity at 200 mμ of the Fab(t) fragment in acid was of the same order of magnitude as the changes in reduced and alkylated normal IgG and myeloma IgG. The Fc(t) fragment showed the highest degree of disorganization of the proteins examined.

In the near ultraviolet region, the circular dichroism bands of all the proteins were reduced significantly by the acid and they were only partially recovered if the measurements were made 30–60 min after neutralization. The rate of diminution and recovery of these bands is likely to be parallel to the change at 202 mμ. The renaturation of all the proteins occurred in almost the same way as of the Fc(t) fragment. Normal IgG and myeloma IgG in which the disulfide bonds were intact precipitated by reneutralization; but the supernatant of the precipitate showed renaturation.

The general circular dichroism patterns observed in acid denaturation of IgG and its fragments, *i.e.*, the shift of the positive band at 202 mμ to a negative band at 200 mμ and relatively little change in the 217-mμ band, were very similar for all of the proteins.

Hydrodynamic Properties of Normal IgG and Its Fragments. The sedimentation coefficients, intrinsic viscosities, and molecular weights of normal IgG and its fragments are listed

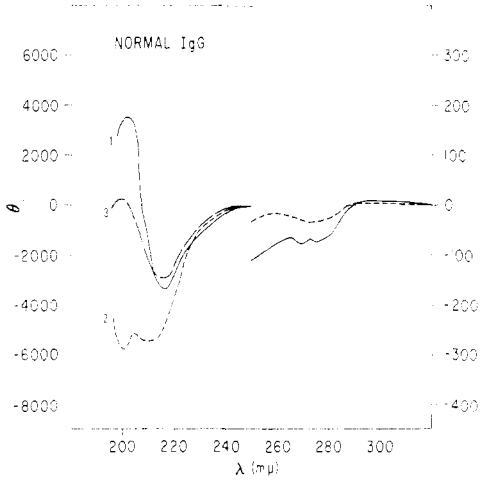

FIGURE 5: The circular dichroism spectra of normal IgG in the far and near ultraviolet. The native state (—, 1) is compared with the pattern in acid (---, 2) and after neutralization of the acid (— — —, 3). The initial neutral solutions contained 0.4–0.6 mg/ml protein in 3 ml of 0.02 M NaCl. The pH of the solutions was adjusted to 2.2 by 0.1 N HCl. The reneutralization was accomplished by adding 100 μl of 0.1 M Na-phosphate, (pH 8.0), and 0.1 N NaOH. The circular dichroism spectra were taken approximately 30 min after preparation of the solutions. The near ultraviolet spectra were measured at 0.5- or 1.0-cm optical path length and the far ultraviolet spectra were recorded at 0.05-cm optical path. Since IgG was precipitated after neutralization, the supernatant solutions were used; the optical path in the deep ultraviolet of these very diluted solutions was 1.0 or 0.5 cm.

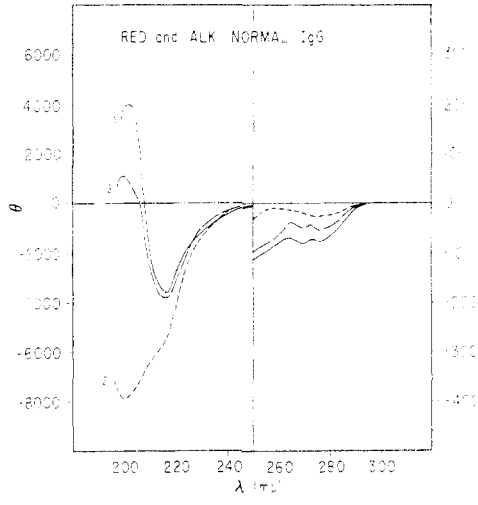

FIGURE 6: Circular dichroism spectra of reduced and alkylated normal IgG in the far and near ultraviolet zones. Designation of the curves and conditions of experiment are the same as in Figure 5.

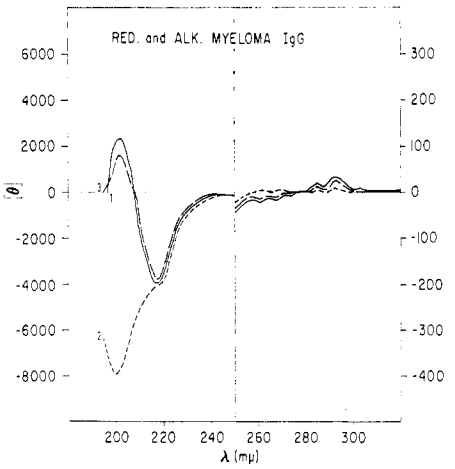

FIGURE 7: Circular dichroism spectra of reduced and alkylated myeloma IgG in the far and near ultraviolet zones. Designation of the curves and conditions of experiment are the same as in Figures 5 and 6.

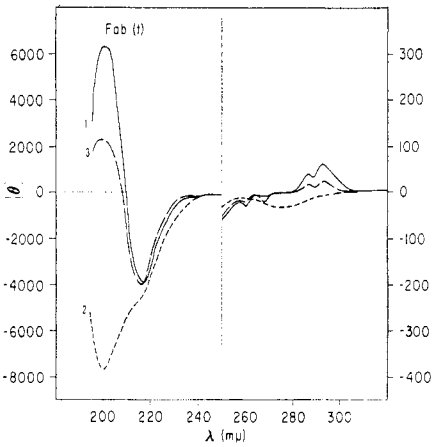

FIGURE 8: Circular dichroism spectra of the fragment Fab(t) in the far and near ultraviolet zones. Designation of the curves and conditions of experiment are the same as in Figures 5, 6, and 7.

in Table II. Since the sedimentation patterns in acid solution indicated inhomogeneity (except of the Fc(t) fragment without salt) the molecular weights are of low precision. However, the decrease of the sedimentation coefficients and the increase in the viscosity observed in acid solution indicate unfolding in accord with the circular dichroism spectra. The Fc(t) fragment was separated into single polypeptide chains in 0.01 N HCl. The Fab(t) fragment appeared to separate incompletely in the same solvent. Also, the incomplete separation of H and L chains was observed in the partially reduced and alkylated IgG solutions in acid as indicated by the molecular weight. In acidic 0.1 M NaCl solution, however, was observed aggregation instead of separation.

Discussion

Tryptic digestion of myeloma IgG was used by Edelman *et al.* (1968) instead of the more commonly used papain digestion because of its restricted substrate specificity. The present work, in which almost the same procedures were applied to normal human IgG, indicated that long digestion produced smaller peptides. Thus a shorter digestion period and more dilute solutions were used to prepare the Fc(t) fragment. Our Fc(t) seemed to be very similar to the Fc(t) obtained by Edelman *et al.* (1968) because it contained the same N-terminal amino acid.

The circular dichroism bands of the human Fc(t) fragment resembled those of the rabbit Fc reported by Cathou *et al.* (1968), but the negative ellipticity at 217 mμ we found was of higher magnitude than the value reported for the rabbit Fc. The difference may be caused by the difference in parent IgGs and methods of cleavage. The Fc of Cathou *et al.* (1968), which had been prepared by the method of Porter (1959) as modified by Nisonoff (1964), might contain peptides of various chain lengths as described by Smith and Utsumi (1967). One of the Fc(t) preparations which we obtained in a longer tryptic digestion time (60 min) showed a lower negative ellipticity at 217 mμ of −1900. The position of the negative circular dichroism band at 217 mμ of our proteins is the same as that of the β structure in poly-L-lysine, as reported *e.g.*, by Sarkar and Doty (1966); however, the amplitude of this peak in the immunoglobulins is much lower than that of the poly-L-lysine in β form. Thus the immunoglobulins probably do not have a very high amount of the β structure. The position of the positive peak of our proteins (202 mμ) is different from that observed in poly-L-lysine in β form (195 mμ, see, *e.g.*, Townend *et al.*, 1966). This discrepancy is difficult to explain, if one wants to assign this 202-mμ band to the β form. It is possible that the shift is caused by side-chain effects and by the specific rigid polypeptide-chain backbone folds which cannot be identified with perfect β structures. Specimens of IgG myeloma globulins and Bence–Jones proteins from different individual sources have shown significant variation in the circular dichroism spectra in the 190–205-mμ zone (Jirgensons, 1969, and unpublished work). While the negative band was observed consistently in all individual samples at 217–218 mμ, more than one positive band appeared in some samples at 192–202 mμ.

The Fc(t) fragment which was denatured by acid yielded a spectrum with a relatively strong negative band at 200 mμ. This position is only a little different from that observed in the typical random coil poly-α-amino acids. Similar negative bands have been reported for alkali-denatured β-lactoglobulin (Townend *et al.*, 1967), reduced soybean trypsin inhibitor (Jirgensons *et al.*, 1969), and of poly-L-lysine film in random conformation (Stevens *et al.*, 1968). According to Stevens *et al.*, this negative band might be expected in the unordered portions of proteins where the conformation is not a fully flexible random coil but is highly constrained by side-chain interactions. A similar explanation could be considered for the present results.

Denaturation of serum γ-globulins by acid has been examined by several authors by means of optical rotation and viscosity (Jirgensons, 1954; Yang and Foster, 1955; Edelhoch *et al.*, 1962; Callaghan and Martin, 1963; Gould *et al.*, 1964), sedimentation behavior (Phelps and Cann, 1957), and fluorescence polarization (Steiner and Edelhoch, 1962). The present method using the far ultraviolet circular dichroism spectrum offers a simple and relatively precise measurement of disorganization. Our viscosity and sedimentation data

support the conclusions based on the circular dichroism spectra.

The circular dichroism spectral titration at 202 mμ indicates that the inflection points are near pH 2.8 (forward titration) and 3.0 (reverse titration). These pH values are far below the pK value (4.73) for the β-and γ-carboxyl groups of IgG reported by Gould *et al.* (1964). This means that disorganization of Fc(t) in acid solution was not the direct result of the disappearance of electrostatic interaction but was due to the appearance of an electrostatic repulsive force which overcame the binding forces. It concurs with the fact that high ionic strength prevented disorganization by acid and separation of the peptide chains. The weakening of the circular dichroism bands in the near ultraviolet suggests the disappearance of organized structures involving the aromatic amino acid residues. The fact that propionic and butyric acids favored the separation of H and L chains (Fleischman *et al.*, 1962) points to the importance of the hydrophobic interactions in interchain bonding.

The incompleteness of reversibility upon neutralization may be due chiefly to the insufficient time allowed for complete renaturation. However, almost complete reversibility was achieved in the case of myeloma globulin in about 30 min after neutralization (Figure 7).

Denaturation with 6 M Gu·HCl produced remarkable changes in the circular dichroism spectra of immunoglobulins and their fragments. Although the denaturation products are not the typical random coils, as also pointed out by Cathou *et al.* (1968) for rabbit IgG, the *decrease* of the negative ellipticity near 217 mμ indicates disorganization of the β structure (Figure 3). In the acid denaturation of IgG and its fragments, a decrease of the negative ellipticity at 217 mμ was never observed. Since the typical random coils of poly-L-lysine and poly-L-glutamate have shown a positive band at 217 mμ (Holzwarth and Doty, 1965; Timasheff *et al.*, 1967), the increase in the negative ellipticity at 217 mμ in acid denaturation can not be explained as a conversion from β form to random coil. A more plausible explanation is that the β structure changed but a little and that some fixed irregular structure which had no circular dichroism band near 217 mμ was affected by the acid. Townend *et al.* (1967) have reported some interesting observations on the alkali denaturation of β-lactoglobulin which very likely has the β structure. In alkaline solution, the circular dichroism spectrum of β-lactoglobulin was dominated by unordered structure but the negative band near 217 mμ was visible as a shoulder. They suggested that this could result from either a native core more resistant to alkali than the other portions of the macromolecule or to the formation of β structure due to aggregation. The former explanation seems to be applicable to the acid denaturation of our proteins at low ionic strength, because sedimentation analyses showed aggregation only at high salt concentrations.

In spite of their diversity in biological activity and composition, the similarity in gross conformation of rabbit IgG fragments was confirmed by Noelken *et al.* (1965). The circular dichroism bands of Fab(t) and Fc(t) fragments are more similar to each other than to the circular dichroism patterns of any other protein. The characteristic changes of the circular dichroism bands in acid also are similar. The results suggest that the conformation in the Fab(t) part of IgG does not differ very much from the conformation in the Fc(t) part.

The effect of the reduction and alkylation of the interchain disulfide bonds in normal and myeloma IgG could not be detected in the circular dichroism spectra in the native state, but it was observed after denaturation with acid (Figures 5 and 6). Hence the presence of interchain disulfide bonds prevented not only separation of the chains but also intramolecular disorganization.

In addition to the mentioned bands, we observed very weak circular dichroism bands of the undenatured fragments Fc(t) and Fab(t) in the 230–245-mμ spectral zone. These bands were not investigated in detail. Cathou *et al.* (1968) have provided detailed information of this spectral zone for rabbit antidinitrophenyl antibody and its fragments. The assignment of these weak effects to definite chromophores, however, appeared to be difficult.

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References

Baumstark, J. S., Laffin, R. J., and Bardawil, W. A. (1964), *Arch. Biochem. Biophys. 108*, 514.

Boyer, P. D. (1954), *J. Amer. Chem. Soc. 76*, 4331.

Callaghan, P., and Martin, N. H. (1963), *Biochem. J. 87*, 225.

Carpenter, F. H. (1967), *Methods Enzymol. 11*, 237.

Cathou, R. E., Kulczycki, A., Jr., and Haber, E. (1968), *Biochemistry 7*, 3958.

Diez, M. J. F., Osuga, D. T., and Feeney, R. E. (1964), *Arch. Biochem. Biophys. 107*, 449.

Edelhoch, H., Lippoldt, R. E., and Steiner, R. F. (1962), *J. Amer. Chem. Soc. 84*, 2133.

Edelman, G. M., Grall, W. E., Waxdal, M. J., and Konigsberg, W. H. (1968), *Biochemistry 7*, 1950.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Fasman, G. D. (1963), *Methods Enzymol. 6*, 928.

Fleischman, J. B., Pain, R. H., and Porter, R. R. (1962), *Arch. Biochem. Biophys., Suppl. 1*, 174.

Gould, H. J., Gill, T. J., and Doty, P. (1964), *J. Biol. Chem. 239*, 2842.

Hamaguchi, K., and Migita, S. (1964), *J. Biochem. (Tokyo) 56*, 512.

Holzwarth, G., and Doty, P. (1965), *J. Amer. Chem. Soc. 87*, 218.

Iizuka, E., and Yang, J. T. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1175.

Ikeda, K., Hamaguchi, K., and Migita, S. (1968), *J. Biochem. (Tokyo) 63*, 654.

Imahori, K. (1963), *Biopolymers 1*, 563.

Imahori, K., and Momoi, H. (1962), *Arch. Biochem. Biophys. 97*, 236.

Jirgensons, B. (1954), *Arch. Biochem. Biophys. 48*, 154.

Jirgensons, B. (1958), *Arch. Biochem. Biophys. 74*, 57.

Jirgensons, B. (1961,) *Arch. Biochem. Biophys. 94*, 59.

Jirgensons, B. (1965), *J. Biol. Chem. 240*, 1064.

Jirgensons, B. (1966a), *J. Biol. Chem. 241*, 147.

Jirgensons, B. (1966b), *Makromol. Chem. 91*, 74.

Jirgensons, B. (1969), Optical Rotatory Dispersion of Proteins

and Other Macromolecules, Berlin, Heidelberg, Springer-Verlag, pp 97, 103.
Jirgensons, B., Kawabata, M., and Capetillo, S. (1969), *Makromol. Chem. 125*, 126.
Kielley, W. W., and Harrington, W. G. (1960), *Biochim. Biophys. Acta 41*, 401.
Li, L. K., and Spector, A. (1969), *J. Amer. Chem. Soc. 91*, 220.
Nisonoff, A. (1964), *Methods Med. Res. 10*, 134.
Noelken, M. E., Nelson, C. A., Buckley, C. E., III, and Tanford, C. (1965), *J. Biol. Chem. 240*, 218.
Phelps, R. A., and Cann, J. R. (1957), *Biochim. Biophys. Acta 23*, 149.
Porter, R. R. (1959), *Biochem. J. 73*, 119.
Quadrifoglio, F., and Urry, D. W. (1968), *J. Amer. Chem. Soc. 90*, 2760.
Ross, D. L., and Jirgensons, B. (1968), *J. Biol. Chem. 243*, 2829.
Sarkar, P. K., and Doty, P. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 981.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.
Scheraga, H. A., and Mandelkern, L. (1953), *J. Amer. Chem. Soc. 75*, 179.
Smith, D. S., and Utsumi, S. (1967), *Nature 216*, 332.
Steiner, R. F., and Edelhoch, H. (1962), *J. Amer. Chem. Soc. 84*, 2139.
Stevens, L., Townend, R., Timasheff, S. N., Fasman, G. D., and Potter, J. (1968), *Biochemistry 7*, 3717.
Timasheff, S. N., Susi, H., Townend, R., Stevens, L., Gorbunoff, M. J., and Kumosinski, T. F. (1967), *in* Conformation of Biopolymers, Vol. 1, Ramachandran, G. N., Ed., New York, N. Y., Academic, p 173.
Townend, R., Kumosinski, T. F., Timasheff, S. N., Fasman, G. D., and Davidson, B. (1966), *Biochem. Biophys. Res. Commun. 23*, 163.
Townend, R., Kumosinski, T. F., and Timasheff, S. N. (1967), *J. Biol. Chem. 242*, 4538.
White, F. H., Jr. (1960), *J. Biol. Chem. 235*, 383.
Winkler, M., and Doty, P. (1961), *Biochim. Biophys. Acta 54*, 448.
World Health Organization (1964), *Bull. W. H. O. 30*, 447.
Yang, J. T., and Foster, J. F. (1955), *J. Amer. Chem. Soc. 77*, 2374.