Circular Dichroism Studies on the Interactions of Haptens with MOPC-315 and MOPC-460 Mouse Myeloma Proteins and Specific Antibodies†

John H. Rockey,* Paul C. Montgomery, Brian J. Underdown, and Keith J. Dorrington

ABSTRACT: The optical activity generated by the noncovalent association of chromophoric haptens and specific immunoglobulins is used as a probe of the antibody combining site. The MOPC-315 and MOPC-460 mouse IgA myeloma proteins, which have substantial affinity for nitrophenyl haptens, are employed as model homogeneous immunoglobulins of related but not identical specificity. Goat and rabbit anti-2,4-dinitrophenyl antibodies also are studied. Extrinsic Cotton effects, observed as induced circular dichroic (CD) bands, are generated when the following ligands with symmetric chromophores are bound within the asymmetric environment of an immunoglobulin combining site: N^{ϵ}-2,4-dinitrophenyllysine, N^{ϵ}-2,4-dinitrophenylaminocaproate, N^{α}-2,4-dinitrophenylglycine, N^{α}-2,4-dinitrophenylglycine methyl ester, N^{ϵ}-2,4,6-trinitrophenylaminocaproate, menadione, 2,4-dinitronaphthol, and 2,4-dinitronaphtholsulfonic acid. Haptens having the same chromophoric group but differing in other structural aspects, as well as haptens differing by virtue of their constituent chromophoric moieties, each generate characteristic CD spectra when complexed with a given protein. The maximum molar ellipticity difference, $([\theta]_{370} - [\theta]_{310})$, of the 2,4,6-trinitrophenylaminocaproate–MOPC-315 protein complex is 6.5×10^4 deg cm^2 dmole^{-1}. Protein-dependent variations in the magnitude, sign, and wavelength maxima of induced CD bands, which are considered to reflect binding site structural differences, are generated when a given hapten is associated with the two mouse myeloma proteins and with specific antibodies, including high and low affinity goat anti-2,4-dinitrophenyl antibodies isolated from a single serum. The induced circular dichroism generated upon binding haptens to reconstituted MOPC-315 protein is identical with that obtained with native protein. The occurrence of large negative induced CD bands centered in the protein aromatic amino acid absorption region near 300 ± 15 nm where bound 2,4-dinitrophenyl and 2,4,6-trinitrophenyl ligands display absorption minima, and the association of extrinsic Cotton effects with strong absorption bands of the haptenic chromophores, in conjunction with red shifts and hypochromic changes in the absorption spectra of bound haptens and the quenching of antibody tryptophan fluorescence by bound ligands, suggest that the optical activity may originate in part by intermolecular dynamic coupling between electronic transitions of the haptenic chromophores and transitions of vicinal tryptophanyl residues of the antibody combining site.

It is well recognized that the binding of low molecular weight chromophoric substances to macromolecules may generate optical activity, observable as extrinsic optical rotatory dispersion (ORD) and circular dichroism (CD) Cotton effects (Blout, 1964; Ulmer and Vallee, 1965; Beychok, 1968). The rotatory power induced by the dissymmetric association of a chromophoric ligand and a protein furnishes an important experimental parameter for analysis of the noncovalent interactions that occur in such complexes and studies of induced optical activity have been used extensively to investigate stereospecific interactions in heme proteins, and between enzymes and coenzymes, substrates, inhibitors, and metal ligands (Ulmer and Vallee, 1965; Urry and Pettegrew, 1967; Nakazawa *et al.*, 1969; Perrin and Hart, 1970; Kägi *et al.*, 1971; Fretto and Strickland, 1971a,b). Extrinsic optical activity also may arise from the noncovalent association of symmetric haptenic chromophores and specific antibodies (Rockey *et al.*, 1971a,b; Conway-Jacobs *et al.*, 1970; Reid *et al.*, 1971). Circular dichroism, because of its high sensitivity to small variations in stereochemical relationships (Tinoco and Cantor, 1970; Hooker and Schellman, 1970), offers a powerful probe with which to compare the three-dimensional structure of combining sites of antibodies of related specificity formed in a single animal, in members of a single species, and in diverse species. In addition, circular dichroism furnishes a useful method for assaying the regain of native combining site structure upon reassociating isolated immunoglobulin heavy- and light-chain subunits.

In the present report we examine the circular dichroism generated by associating a range of chromophoric haptens with two homogeneous mouse IgA myeloma proteins having substantial affinity for haptens (Eisen *et al.*, 1968; Jaffe *et al.*, 1971; Underdown *et al.*, 1971), and with goat and rabbit antibodies. Both hapten-dependent and protein-dependent CD spectral features are defined. The recovery of native binding site structure in a reconstituted homogeneous immunoglobulin is demonstrated by circular dichroism.

† From the Division of Immunochemistry, Department of Ophthalmology, School of Medicine, and the Department of Microbiology, School of Dental Medicine and Center for Oral Health Research, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and the Departments of Biochemistry, Medicine and Medical Cell Biology, Institute for Immunology, University of Toronto, Toronto 5, Canada. *Received July 30, 1971.* The initial phase of this work was completed at the MRC Molecular Pharmacology Unit, Medical School, Cambridge, England. This work was supported by Research Grants AI-05305 from the National Institute of Allergy and Infectious Diseases, and DE-02623 from the National Institute of Dental Research, U. S. Public Health Service, Department of Health, Education and Welfare; by Grants MA-4259 and MA-4050 from the Medical Research Council of Canada; and by the Medical Research Council of Great Britain. Presented in part before the American Association of Immunologists, Chicago, Ill., April 14, 1971 (Rockey *et al.* (1971)).

* Author to whom correspondence should be addressed at the Department of Ophthalmology, School of Medicine, University of Pennsylvania.

Materials and Methods

Purification of MOPC-315 and MOPC-460 Proteins. The mouse plasmacytoma MOPC-315 and MOPC-460 tumor lines (Potter and Lieberman, 1967; Eisen *et al.*, 1968; Jaffe *et al.*, 1971) were obtained from Dr. Herman N. Eisen, and maintained in BALB/c mice. MOPC-315 and MOPC-460 IgA myeloma proteins were purified by affinity chromatography utilizing a solid immunoabsorbant of Dnp-lysine[1] covalently linked to Sepharose-2B (Dnp-lysine-Sepharose) (Porath *et al.*, 1967; Rockey *et al.*, 1971a,b). In some instances, proteins were reduced with 10 mM dithiothreitol at pH 8.4 for 1 hr at 25°, and alkylated at 0° by the addition of solid iodoacetamide (12 mM) prior to affinity chromatography. MOPC-315 and MOPC-460 IgA myeloma protein concentrations were determined from the absorbence at 280 nm (corrected for light scattering) employing extinction coefficients, $\epsilon_{1\,cm}^{1\,mg/ml}$, of 1.44 and 1.55, and molecular weights of 153,000 and 150,000, respectively (Haimovich *et al.*, 1970; Jaffe *et al.*, 1971; Underdown *et al.*, 1971).

Preparation of Goat and Rabbit Anti-Dnp Antibodies. Goats and rabbits were immunized by subcutaneous injection of Dnp antigens in complete Freund's adjuvant. Antiserum B-1 was obtained from a goat that had received a primary injection of 10 mg of Dnp_{50}-BγG followed by an identical antigen injection 2 months later. Antiserum D-O (kindly provided by E. S. Simms, Washington University) was obtained from a goat that had received 2 injections of 25 mg of Dnp-hemocyanin followed a year later by two injections of 25 mg of Dnp_{60}-BγG. The goats were bled 2 weeks after the last antigen administration. The antisera contained 1–2 mg of anti-Dnp antibody/ml. Anti-Dnp antibodies of antiserum D-O were purified by affinity chromatography. Antibodies were eluted from the Dnp-lysine-Sepharose immunosorbant with 0.1 M dinitrophenol in 0.15 M NaCl–10 mM phosphate buffer (pH 7.2), and the dinitrophenol hapten was removed by chromatography on a column of Dowex 1 (Cl^- form, 20–50 mesh). The yield was 60% of the antibody precipitable from D-O antiserum with Dnp_{35}-HSA. Approximately 8% of the antibody combining sites was occupied by residual hapten.

Anti-Dnp antibodies of antiserum B-1 were fractionated into two subsets by affinity chromatography. Fraction B-1 (high K) was obtained by passing an excess of the B-1 serum over a Dnp-lysine-Sepharose column under conditions such that approximately 80% of the antibodies was not bound by the column. The higher affinity antibodies which were bound were eluted with 0.1 M dinitrophenol and the hapten was removed by chromatography on Dowex 1 (Cl^-). The yield of fraction B-1 (high K) was 10% of the total antibody precipitable from antiserum B-1 with Dnp_{35}-HSA. Fraction B-1 (low K) was obtained by precipitating the residual antibodies, not bound by the Dnp-lysine-Sepharose immunosorbent during isolation of fraction B-1 (high K), with Dnp_{35}-HSA. The washed specific precipitate was dissolved in 0.1 M dinitrophenol–0.15 M NaCl–10 mM phosphate buffer (pH 7.2), and the anti-Dnp antibody was purified by chromatography on a double-bed column of DEAE-cellulose Dowex 1 according to Eisen *et al.* (1967). The yield of fraction B-1 (low K) was 60% of the total precipitable anti-Dnp antibody present in antiserum B-1. The percentage of combining sites occupied by residual hapten was approximately 1% for both fractions of antiserum B-1.

Fluorescence quenching Q_{max} values were 0.73 for all three goat anti-Dnp antibody preparations. Anti-Dnp antibody was isolated in a similar manner by specific immunoprecipitation with Dnp_{35}-HSA, from rabbits hyperimmunized with Dnp-BγG in complete Freund's adjuvant. Antibody concentrations were determined from the absorbance at 280 nm, employing an extinction coefficient, $\epsilon_{1\,cm}^{1\,mg/ml}$, of 1.5 and a molecular weight of 150,000.

Ligands. Dnp-lysine (N^ϵ-2,4-dinitrophenyl-L-lysine), Dnp-aminocaproate (N^ϵ-2,4-dinitrophenylaminocaproate), Dnp-glycine (N^α-2,4-dinitrophenylglycine), and Dnp-tryptophan (N^α-2,4-dinitrophenyl-L-tryptophan) were obtained from Sigma Chemical Co. Tnp-aminocaproate (N^ϵ-2,4,6-trinitrophenylaminocaproate) was synthesized as previously described (Rockey *et al.*, 1971b). Dnp-glycine methyl ester (N^α-2,4-dinitrophenylglycine methyl ester) was synthesized by refluxing Dnp-glycine with methanol and thionyl chloride. Dnp-glycine (270 mg) dissolved in 22 ml of methanol was cooled to −10°, and 7.7 ml of thionyl chloride (reagent grade, Fisher Scientific) was added. The mixture was incubated at 40° for 3 hr, refluxed for 3 hr, maintained at 20° for 16 hr, and flash evaporated. Dnp-glycine methyl ester was recrystallized three times from methanol–water. High-voltage electrophoresis on silica gel plates in pyridine–acetic acid water (26:1:973), pH 6.5, demonstrated that the Dnp-glycine methyl ester was free of residual Dnp-glycine. Menadione (2-methyl-1,4-naphthoquinone) and 2,4-dinitro-1-naphthol were obtained from Eastman Organic Chemicals, and 2,4-dinitronaphtholsulfonic acid (2,4-dinitro-1-naphthol-7-sulfonic acid) from Fisher Scientific. Molar extinction coefficients employed to determine ligand concentrations were: Dnp-lysine, ϵ_{362} 1.75×10^4; Dnp-aminocaproate, ϵ_{362} 1.75×10^4; Tnp-aminocaproate, ϵ_{348} 1.57×10^4; Dnp-glycine, ϵ_{360} 1.59×10^4; Dnp-glycine methyl ester, ϵ_{360} 1.59×10^4; menadione, ϵ_{340} 0.2×10^4; 2,4-dinitronaphthol, ϵ_{395} 1.46×10^4; 2,4-dinitronaphtholsulfonic acid, ϵ_{395} 1.46×10^4; Dnp-tryptophan, ϵ_{360} 1.70×10^4.

Circular Dichroism. Circular dichroism (CD) was measured at 4–37° with a Jasco Model ORD/UV-5 spectropolarimeter equipped with a circular dichroism attachment, a Durrum-Jasco Model J-10 circular dichrometer, or a Cary Model 61 circular dichrometer. The instruments were calibrated with *d*-10-camphorsulfonic acid in water, and *d*-camphor in methanol, according to Cassim and Yang (1969). Fused quartz cells with path lengths of from 2.0 cm to 0.1 mm and protein concentrations ranging from 5×10^{-3} g to 1 g/100 ml were used in determining the CD spectra of antibody and antibody–ligand complexes between 600 and 185 nm. The data are presented in terms of molar ellipticities, $[\theta]_\lambda$, in deg cm^2 dmole^{-1}, where $[\theta]_\lambda = (2.303)(4500/\pi)(\epsilon_l - \epsilon_r)_\lambda$. The molar dichroic difference absorptivity, $(\epsilon_l - \epsilon_r)_\lambda = (A_l - A_r)_\lambda/lc$, where l is the cell path length in centimeters, c is the molar concentration of protein residue or bound ligand, and $(A_l - A_r)_\lambda$ is the observed difference in absorption between left and right circularly polarized light at wavelength λ. Data also are presented in terms of the observed ellipticity, $\theta_\lambda = 3298(A_l - A_r)_\lambda$.

Binding curves were constructed by serially adding hapten to a constant amount of antibody and measuring the change in circular dichroism. The data are plotted as θ_λ *vs.* [total ligand concentration] and as $[\theta]_\lambda$ (apparent) *vs.* [total ligand concentration], where $[\theta]_\lambda$(app) = θ_λ[total ligand concentra-

[1] Abbreviations used are: Dnp, 2,4-dinitrophenyl; Tnp, 2,4,6-trinitrophenyl; BγG, bovine IgG-globulin; HSA, human serum albumin; Dnp_{50}-BγG and Dnp_{35}-HSA have indicated number of Dnp groups (substituted on lysine residues) per molecule of protein; Dnp-lysine-Sepharose, immunoabsorbant of Dnp-lysine covalently linked to Sepharose-2B.

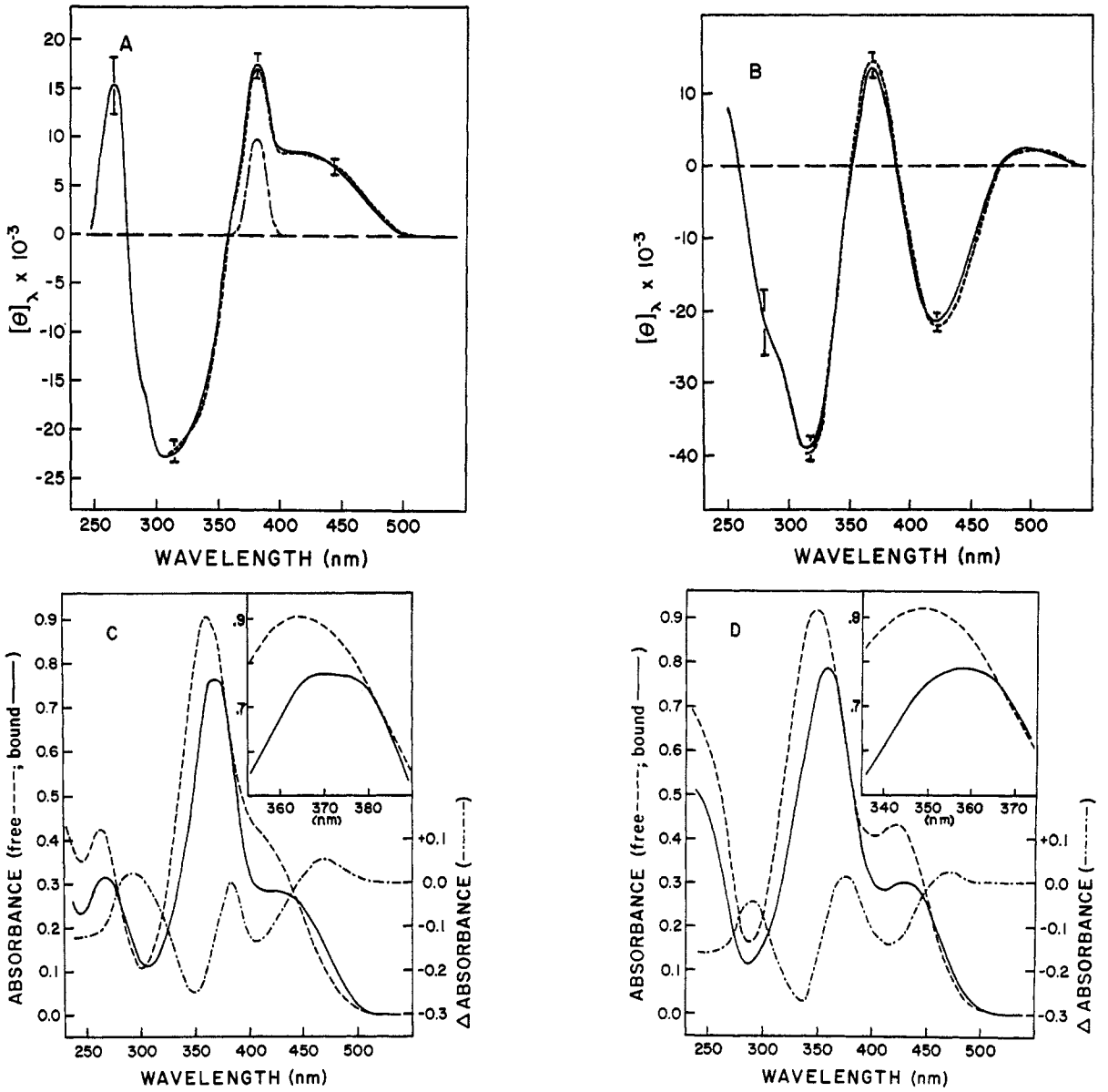

FIGURE 1: (A) Circular dichroic difference spectrum of Dnp-aminocaproate complexed with MOPC-315 mouse IgA myeloma protein *vs.* MOPC-315 protein (———). The ellipticity of the MOPC-315 protein in the absence of added hapten was subtracted algebraically from that of the MOPC-315 protein–Dnp-aminocaproate complex to give the induced CD spectrum below 325 nm. Dnp-aminocaproate in free solution did not display circular dichroism (— — —). Molar ellipticities, $[\theta]_\lambda$, were determined by constructing a CD hapten binding curve. A corrected 382-nm CD band (——·——·——) was obtained by subtracting the lower wavelength extension of the broad CD band centered near 410–420 nm from the observed molar ellipticity. The circular dichroic difference spectrum of Dnp-aminocaproate complexed with reconstituted MOPC-315 protein, formed by reassociating isolated heavy- and light-chain subunits, also is presented (·····). Solvent 0.1 M NaCl–10 mM phosphate buffer (pH 7.5). Spectra examined at 27° in a Cary 61 circular dichrometer with the slit width programmed to maintain a bandwidth of 1.5 nm at all wavelengths (240–550 nm). The vertical bars represent the signal to noise ratios in different wavelength regions of the spectrum. (B) Circular dichroic difference spectrum of Tnp-aminocaproate complexed with MOPC-315 protein *vs.* MOPC-315 protein (———). Tnp-aminocaproate in free solution did not display circular dichroism (— — —). A positive CD band centered below 250 nm was seen in the induced CD spectrum. The CD difference spectrum of Tnp-aminocaproate bound to reconstituted MOPC-315 protein also is recorded (·····). (C) Absorption spectra, between 240 and 550 nm, of Dnp-aminocaproate in free solution (— — — —), and of an equal molar concentration of Dnp-aminocaproate complexed with the mouse MOPC-315 protein (———). An equal concentration of the MOPC-315 protein in the absence of added hapten was used as reference to obtain the spectrum of bound Dnp-aminocaproate. The Dnp-aminocaproate–MOPC-315 protein *vs.* MOPC-315 protein difference spectrum was examined at $r = 1.1$–1.3 where more than 97–99% of the total hapten present is specifically bound to the MOPC-315 protein ($K_0 = 0.6 \times 10^7$ M^{-1}; $a = 0.98$). Free Dnp-aminocaproate, $\epsilon_{362\text{ nm}}$ 1.75×10^4; bound Dnp-aminocaproate, $\epsilon_{370\text{ nm}}$ 1.50×10^4. The spectra at maximum absorbance are plotted on an expanded wavelength scale in the upper right insert to illustrate that the bound Dnp-aminocaproate spectrum is composed of more than a single component near λ_{max}. A similar observation was made when the absorption spectrum of Dnp-lysine complexed with MOPC-315 protein was examined. The absorption difference spectrum (Δ absorbance) of bound *vs.* free Dnp-aminocaproate also is plotted (——·——). (D) Absorption spectra of Tnp-aminocaproate in free solution (— — — — — —); and of an equal molar concentration of Tnp-aminocaproate bound to the MOPC-315 protein ($r = 1.0$–1.3) *vs.* MOPC-315 protein (———). Free Tnp-aminocaproate, $\epsilon_{348\text{ nm}}$ 1.57×10^4; bound Tnp-aminocaproate, $\epsilon_{359\text{ nm}}$ 1.34×10^4. The bound *vs.* free Tnp-aminocaproate difference spectrum also is presented (——·——).

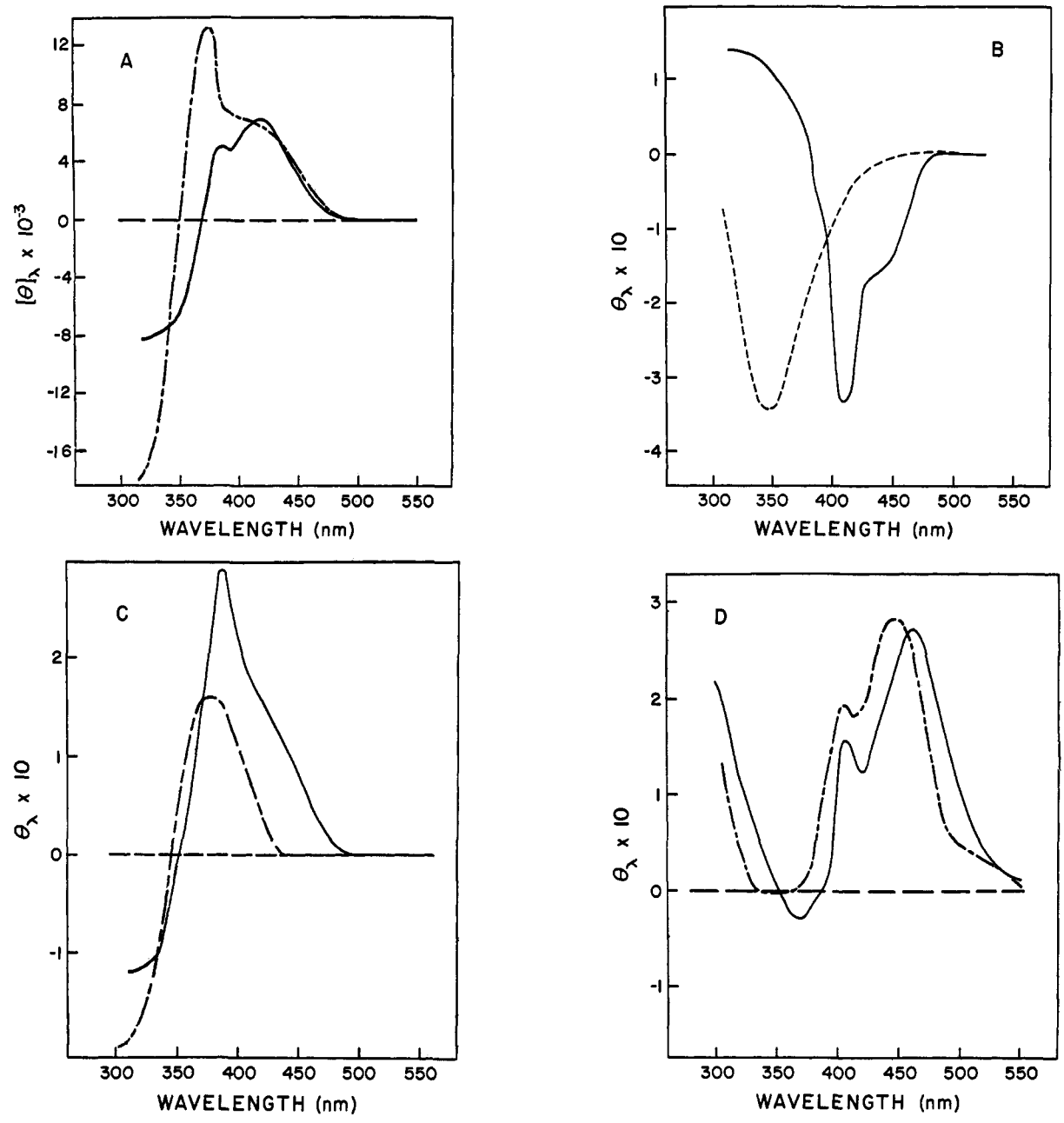

FIGURE 2: (A) Circular dichroic difference spectra of Dnp-glycine (——) and Dnp-glycine methyl ester (——·——) complexed with mouse MOPC-315 IgA protein *vs.* the MOPC-315 protein alone. Molar ellipticities, $[\theta]_\lambda$, were determined by constructing CD hapten binding curves. The maximum molar ellipticity differences, ($[\theta]_{420} - [\theta]_{325}$) or ($[\theta]_{373} - [\theta]_{325}$), were obtained as the tangent of the slope of the observed ellipticity difference *vs.* [total ligand concentration] plots at infinite ligand dilution. Dnp-glycine and Dnp-glycine methyl ester in free solution did not display circular dichroism (— — — — —). (B) Induced circular dichroic difference spectra of menadione (— — —) and 2,4-dinitronaphthol (———) complexed with the mouse MOPC-315 IgA protein *vs.* the MOPC-315 protein alone. Observed ellipticity, θ_λ, plotted against wavelength. (C) Circular dichroic spectra of Dnp-aminocaproate or Tnp-aminocaproate in free solution (—— ——); and of Dnp-aminocaproate (———) or TNP-aminocaproate (——·——) complexed with MOPC-460 mouse IgA myeloma protein, less the circular dichroism of the MOPC-460 protein alone. Observed ellipticity plotted *vs.* wavelength. (D) Circular dichroic spectra of 2,4-dinitronaphthol or 2,4-dinitronaphtholsulfonic acid in free solution (—— ——); and of 2,4-dinitronaphthol (——) or 2,4-dinitronaphtholsulfonic acid (———·———) complexed with the mouse MOPC-460 IgA myeloma protein, less the circular dichroism of the MOPC-460 protein alone.

tion]$^{-1}$ l^{-1}. When the total ligand concentration was greater than the molar concentration of antibody combining sites, the combining site concentration was used to calculate $[\theta]_\lambda$ (app). The molar ellipticities of haptens bound to homogeneous proteins were obtained from the slope of the θ_λ *vs.* [total ligand concentration] plots where the percentage of the total ligand that was in free solution was minimal. With homogeneous high-affinity protein–hapten systems, the θ_λ *vs.* [total ligand concentration] data in the region below antibody site saturation were treated by the method of least squares to give $[\theta]_\lambda$. With homogeneous low-affinity protein–hapten systems, where significant free hapten is present at high r values, the tangent of the θ_λ *vs.* [total ligand concentration] plot at infinite ligand dilution was taken to give $[\theta]_\lambda$ for bound hapten (Rockey *et al.*, 1971a,b). The rotational strength, R, uncorrected for solvent polarizability, was obtained from the relationship $R = 0.696 \times 10^{-42}(\pi)^{1/2}[\theta]_{\lambda_{max}}(\Delta\lambda/\lambda_{max})$, where $[\theta]_{\lambda_{max}}$ is the maximum molar ellipticity of a CD band, λ_{max}

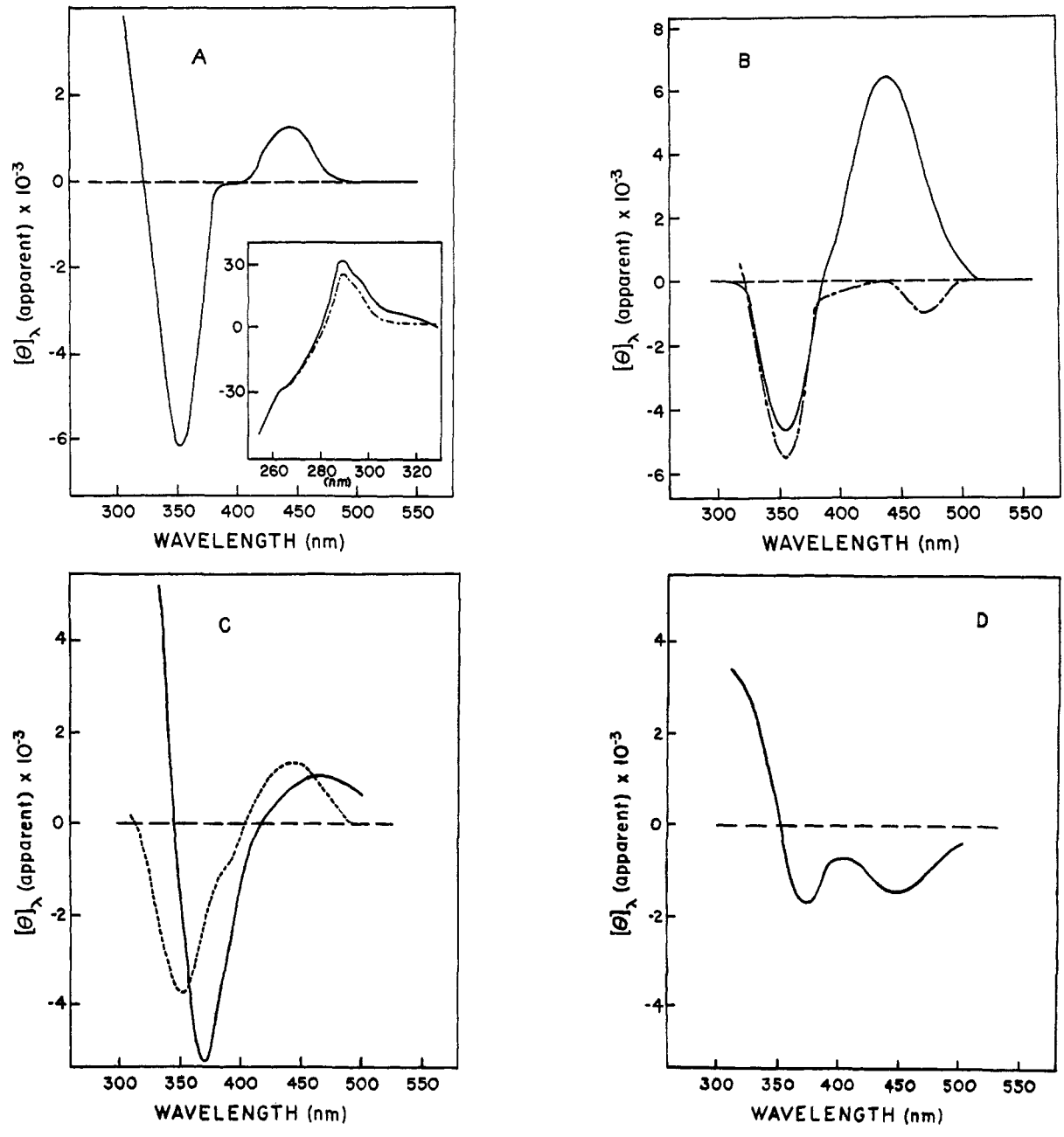

FIGURE 3: (A) Circular dichroic spectra of Dnp-lysine in free solution (— —); and of Dnp-lysine complexed with goat anti-Dnp antibody D-O less the circular dichroism of the antibody alone (———). Antibody concentration 4.45 × 10^{-5} M; Dnp-lysine concentration 1.03 × 10^{-4} M. $[\theta]_\lambda$(app) values calculated by assuming that the concentration of bound hapten equaled the molar concentration of antibody combining sites (8.9 × 10^{-5} M). The insert presents the circular dichroic spectra of the Dnp-lysine goat anti-Dnp antibody complex (———), and of an equal molar concentration of goat anti-DNP antibody in the absence of hapten (—— · ——), measured in a 0.1-cm path-length cell, in the region of high absorptivity by protein aromatic amino acid residues. (B) Induced circular dichroic difference spectra of goat anti-Dnp antibody D-O (3.71 × 10^{-5} M) plus Tnp-aminocaproate (3.43 × 10^{-5} M) *vs.* goat anti-Dnp antibody alone (——); and of goat anti-Dnp antibody D-O (4.45 × 10^{-5} M) plus Dnp-glycine (0.92 × 10^{-4} M) *vs.* antibody alone (—— — ———). Tnp-aminocaproate or Dnp-glycine in free solution (— — —). (C) Circular dichroic difference spectra, generated by goat anti-Dnp antibodies with different average intrinsic association constants (Table I), separated by affinity chromatography from antiserum B-1. Higher affinity goat anti-Dnp antibody B-1 (high K) (2.93 × 10^{-5} M) plus Dnp-lysine (1.03 × 10^{-4} M) (— — — — — —). Lower affinity goat anti-Dnp antibody B-1 (low K) (4.68 × 10^{-5} M) plus Dnp-lysine (1.03 × 10^{-4} M) (———). The circular dichroism of the antibody alone has been algebraically subtracted to give the induced CD difference spectra. Dnp-lysine in free solution (——————). (D) Circular dichroic difference spectrum of Dnp-glycine (0.92 × 10^{-4} M) complexed with lower affinity goat anti-Dnp antibody B-1 (low K) (4.68 × 10^{-5} M) (——). Dnp-glycine in free solution (— — —).

is the wavelength at maximum ellipticity, and $\Delta\lambda$ is the wavelength interval at 0.368 of $[\theta]_{\lambda_{max}}$ (Moscowitz, 1960).

Absorption Spectroscopy. Absorption spectra and absorptivity difference spectra were determined at 25° with either a Jasco ORD/UV5 recording spectrophotometer, a Zeiss PM QII spectrophotometer or a Cary Model 15 recording spectrophotometer.

Fluorescence Quenching. The quenching of antibody tryptophan fluorescence by bound hapten was measured at 25–28° in the thermostatted cell compartment of either a Zeiss or

an Aminco-Bowman spectrophotofluorometer (Velick *et al.*, 1960; Rockey, 1967; Eisen and McGuigan, 1971). Maximum fluorescence quenching (Q_{max}) was determined by titration with an excess of Dnp-lysine. Fluorescence data were corrected for dilution and internal quenching as previously described (Rockey, 1967; Jaffe *et al.*, 1971) and used to construct hapten binding curves. Average intrinsic association constants, K_0, were determined from the plots of r/c' *vs.* r, where r is the moles of hapten bound per mole of antibody, and c' is the free hapten concentration. The valence of the antibody was taken as 2 ($n = 2$). The binding data also were analyzed with the assistance of a computer program (Dammkoehler and Gallagher, 1971) in terms of the Sips distribution function, $\log [r/(n - r)] = a \log c' + a \log K_0$, where a is the index of heterogeneity (Wu and Rockey, 1969; Eisen and McGuigan, 1971).

Reassociation of MOPC-315 Protein Subunits. MOPC-315 protein at a concentration of 2 mg/ml was reduced with 10 mM dithiothreitol and alkylated with iodoacetamide, transferred at 4° to 1 M propionic acid by Sephadex G-25 gel filtration, and immediately applied to a Sephadex G-100 column equilibrated with 1 M propionic acid. The column was eluted with the same solvent and the resolved monomeric heavy- and light-chain fractions were immediately and exhaustively dialyzed at 4° against 4 mM sodium acetate buffer (pH 5.4) and recombined in the same solvent (Stevenson, 1968; Stevenson and Dorrington, 1970). The recombined protein was dialyzed against 0.1 M NaCl–10 mM phosphate buffer (pH 7.5) and filtered at 4° through 4 in-series columns (each column 2 × 60 cm) of Sephadex G-150 in the same solvent, and the 7S fraction was used for CD ligand binding studies.

Results

MOPC-315 Protein–Hapten Complexes. The circular dichroic spectrum of the MOPC-315 mouse IgA myeloma protein displayed a large positive CD band at 200 nm, a large negative band at 217 nm, and small negative CD bands at 260, 267, 285, and 293, and near 252, 277, and 300 nm, but no optical activity at wavelengths greater than 325 nm. Extrinsic Cotton effects generated by associating Dnp-aminocaproate, Tnp-aminocaproate, Dnp-glycine, Dnp-glycine methyl ester, menadione, and 2,4-dinitronaphthol with protein MOPC-315 are illustrated in Figures 1 and 2. Extension of the induced CD spectra below 325 nm was accomplished by algebraically subtracting the circular dichroism of the protein alone from that of the protein–hapten complexes. The large negative CD bands below 350–360 nm in the Dnp- and Tnp-hapten–MOPC-315 protein spectra (Figure 1A,B) were centered near 290–315 nm. The lower signal to noise ratio resulting from the high absorptivity of protein aromatic amino acid residues below 310 nm made assignment of wavelength maxima (λ_{max}) for induced CD bands centered here less certain. Absorption spectra of free and bound Dnp-aminocaproate and Tnp-aminocaproate, and difference spectra of bound *vs.* free ligands, between 240 and 550 nm, are presented in Figure 1C,D. Absorption spectra and induced CD spectra of Dnp-lysine complexed with protein MOPC-315 were virtually identical with those obtained with Dnp-aminocaproate.

To determine the effect of temperature variations on the induced circular dichroic spectra, complexes of Tnp-aminocaproate and Dnp-aminocaproate with protein MOPC-315 were examined in hapten excess at 4, 25, and 37°. Increasing the temperature from 4 to 37° resulted in a 4–9% decrease in the observed ellipticity at wavelength maxima (λ_{max}), but no change in the λ_{max} of individual CD bands.

TABLE I: Average Intrinsic Association Constants (K_0) and Indices of Heterogeneity (a) of Goat Anti-Dnp Antibodies.

Antibody Prepn[a]	Ligand	$K_0 \times 10^{-7}$ (l./Mole)	a
D-O	Dnp-lysine	1.3	0.69
	Dnp-glycine	0.13	0.71
	Tnp-aminocaproate	0.02	0.71
B-1 (high K)[b]	Dnp-lysine	0.94	0.79
	Dnp-glycine	0.30	0.46
	Tnp-aminocaproate	0.07	0.39
B-1 (low K)[b]	Dnp-lysine	0.21	0.64
	Dnp-glycine	0.07	0.76
	Tnp-aminocaproate	0.03	0.53

[a] The three goat anti-Dnp preparations contained 7S IgG antibodies alone when examined by Sephadex G-200 gel filtration and immunoelectrophoresis. [b] Goat anti-Dnp antibodies of higher and lower affinity separated by affinity chromatography from serum of a single bleeding of goat B-1.

Reconstituted MOPC-315 Protein. The extrinsic Cotton effects that were generated by binding Dnp- and Tnp-haptens to reconstituted protein, formed by reassociating isolated MOPC-315 IgA heavy and light chains, were closely similar to those obtained with the nonreduced protein and with MOPC-315 protein isolated by affinity chromatography after mild reduction and alkylation (Figure 1). The circular dichroic spectrum of the reconstituted protein alone also was closely similar to that of native protein (J. H. Rockey and R. M. Freed, unpublished observations).

MOPC-460 Protein–Hapten Complexes. Induced circular dichroic bands may vary in magnitude, sign, and wavelength maxima (λ_{max}) when a single chromophoric ligand is bound by two distinct homogeneous proteins of related but not identical specificity. The CD spectra of Dnp-aminocaproate, Tnp-aminocaproate, 2,4-dinitronaphthol, and 2,4-dinitronaphtholsulfonic acid complexed with the MOPC-460 mouse IgA myeloma protein are presented in Figure 2C,D and differ from those obtained with MOPC-315 protein. The Dnp-aminocaproate–MOPC-460 protein spectrum displays a positive CD maximum at 388 nm (*cf.* positive CD band at 382 nm in Dnp-aminocaproate–MOPC-315 protein spectrum, Figure 1). The CD spectrum of Dnp-lysine bound to MOPC-460 protein was closely similar to the Dnp-aminocaproate–MOPC-460 protein spectrum. The induced circular dichroism of the 2,4-dinitronaphthol–MOPC-460 protein complex, in hapten excess, was unchanged when examined at temperatures ranging from 4 to 37°.

Goat Anti-Dnp Antibody–Hapten Complexes. Extrinsic Cotton effects obtained with three goat anti-Dnp antibody preparations and Dnp-lysine, Tnp-aminocaproate, and Dnp-glycine are illustrated in Figure 3. The average intrinsic association constants (K_0) and the indices of heterogeneity (a) for these antibodies are recorded in Table I. Preparations B-1 (high K) and B-1 (low K) were separated by affinity chromatography from a single serum. The induced CD bands generated with B-1 (high K) and Dnp-lysine differed in magnitude and wavelength maxima from those generated with B-1 (low K) (Figure 3C).

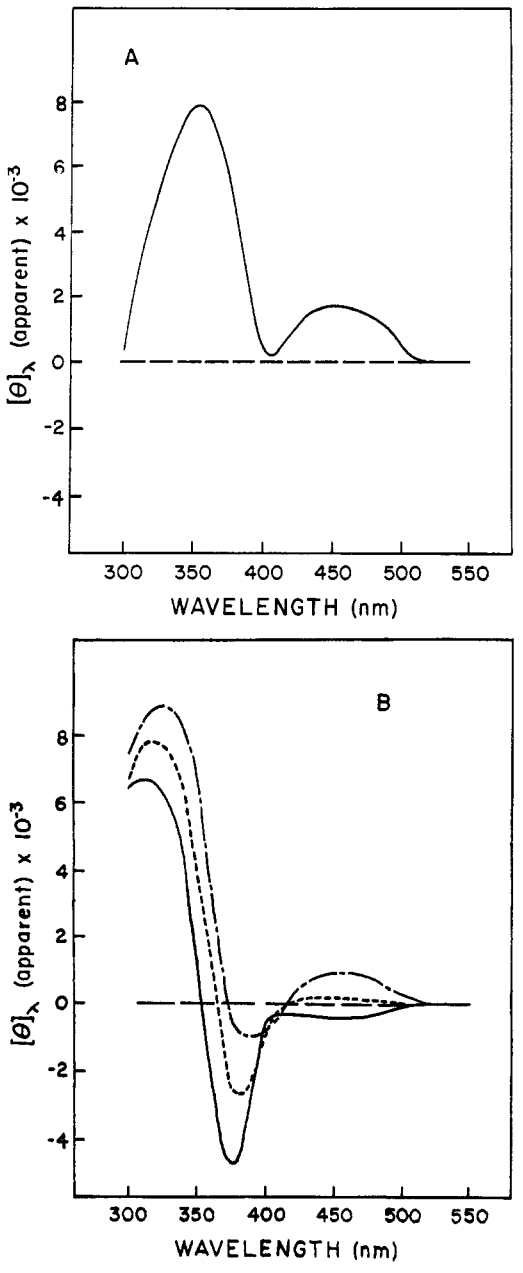

FIGURE 4: (A) Induced circular dichroic difference spectra of rabbit anti-Dnp antibody (3.0×10^{-5} M) plus Dnp-lysine (1.0×10^{-4} M) *vs.* rabbit anti-Dnp antibody alone (———). (B) Induced CD spectra of Dnp-aminocaproate complexed with a second rabbit anti-Dnp antibody preparation (3.0×10^{-5} M). The induced CD spectra obtained upon sequential addition of hapten (3.0×10^{-5} M, ———; 4.5×10^{-5} M, — — — —; 1.1×10^{-4} M, —— — ——) to this heterogeneous antibody preparation varied substantially in the relative ellipticities at different wavelengths and did not show an isosbestic crossover point (crossover varied from 350 to 370 nm). At higher hapten concentrations, the negative ellipticity at 380 nm decreased, and a positive CD band centered near 430 nm developed.

TABLE II: Induced Circular Dichroism of Antibody Hapten Complexes.

Antibody	Hapten	λ_{max} (nm)	$[\theta]_\lambda{}^a$ ($\times 10^{-3}$)	R^b ($\times 10^{39}$)	Antibody	Hapten	λ_{max} (nm)	$[\theta]_\lambda{}^a$ ($\times 10^{-3}$)	R^b ($\times 10^{39}$)
MOPC-315	N^ϵ-Dnp-lysine	300	−23.7		Goat anti-DNP(D-O)	N^ϵ-Dnp-lysine	300	+5.6[d]	+0.7
		382	+16.6				352	−6.2[d]	−0.2
		410	+8.1				441	+1.2[d]	
	N^ϵ-Dnp-aminocaproate	300	−25.0	−6.9		N^ϵ-Tnp-aminocaproate	355	−4.5[d]	
		382	+16.8				440	+6.4[d]	
		382	+10.0[c]	+0.6					
		410	+8.2			N^α-Dnp-glycine	354	−5.3[d]	
	N^ϵ-Tnp-aminocaproate	310	−48.7	−13.9			468	−1.0[d]	
		370	+16.5	+1.5	Goat anti-DNP(B-1) (low K)	N^ϵ-Dnp-lysine	370	−5.2[d]	
		423	−27.9						
	N^α-Dnp-glycine	300	−7.9	−2.8			465	+1.1[d]	
		380	+4.8						
		380	+2.4[c]	+0.2		N^α-Dnp-glycine	375	−1.6[d]	
		420	+6.8				445	−1.4[d]	
	N^α-Dnp-glycine methyl ester	300	−19.5	−6.0	Goat anti-DNP(B-1) (high K)	N^ϵ-Dnp-lysine	352	−3.9[d]	
		373	+13.7				445	+1.4[d]	
		373	+6.7[c]	+0.4	Rabbit anti-DNP	N^ϵ-Dnp-lysine	354	+8.0[d]	
		410	+6.8				455	+1.7[d]	

[a] Molar ellipticity, $[\theta]_\lambda = (2.303)(4500/\pi)(\epsilon_l - \epsilon_r)_\lambda$, in deg cm^2 dmole^{-1}, at wavelength λ. [b] Rotational strength, in cgs units, uncorrected for solvent polarizability. [c] Corrected for ellipticity contributed by extension of broad CD band centered near 410–420 nm. [d] Apparent $[\theta]_\lambda$ values, calculated by assuming that the molar concentration of bound ligand equaled the total ligand concentration (examined in antibody excess), or the molar concentration of antibody combining sites (examined in hapten excess).

Rabbit Anti-Dnp Antibody–Hapten Complexes. Extrinsic Cotton effects generated by binding Dnp-haptens to rabbit anti-Dnp antibodies are illustrated in Figure 4.

Hapten Binding Curves. Circular dichroism binding studies furnish additional parameters with which to evaluate the homogeneity of an antibody preparation. The induced CD spectrum of each of the MOPC-315 and MOPC-460 protein–hapten complexes was examined over a wide range of hapten concentrations (r = 0.1–2.0). Each generated an unvarying induced CD spectrum which was independent of the proportion of the total antibody combining sites occupied by hapten, with constant wavelength maxima (λ_{max}) for CD bands, unchanging (isosbestic) cross-over points and constant ratios between the ellipticities at each wavelength across the induced CD spectum, reflecting the homogeneity of the MOPC-315 and MOPC-460 proteins. In contrast, induced CD spectra generated by the sequential addition of haptens to heterogeneous anti-DNP antibody preparations (Figure 4) varied in the wavelength maxima of CD bands, the ratio of induced ellipticities at different wavelengths and/or in the wavelengths of crossover points when different fractions of the total antibody combining site population were occupied by hapten. A summary of induced molar ellipticity values for hapten–antibody complexes and rotational strengths calculated for selected circular dichroic bands are recorded in Table II.

Discussion

Extrinsic Cotton effects arising from the noncovalent association of haptenic chromophores and antibodies provide a powerful probe of the antibody combining site, capable of detecting small structural differences between binding sites of closely similar specificity. The optical activity of the inherently symmetric chromophoric moiety of the bound ligand is generated by interactions with asymmetrically situated vicinal groups of the protein (Eyring *et al.*, 1968; Tinoco and Cantor, 1970; Hsu and Woody, 1971), and induced circular dichroic spectra reflect alterations in stereochemical relationships and amino acid replacements within individual binding sites. The mouse MOPC-315 and MOPC-460 IgA proteins are well suited as model immunoglobulins for comparative studies because of their homogeneity, their related specificities and their affinities for a number of chromophoric ligands (Eisen *et al.*, 1968; Jaffe *et al.*, 1971). The availability of several haptens, each of which generate distinct CD spectral features when bound by a given protein, increases the power of circular dichroism to demonstrate small differences between immunoglobulin combining sites. With regard to the nature of the ligand, two approaches used here have led to hapten-dependent CD spectral differences. Haptens that differed by virtue of their constituent chromophoric moieties generated unique CD spectra when bound by a homogeneous protein. Distinctive CD spectra also were obtained when haptens possessing the same dinitrophenyl chromophore but differing in other structural features were complexed with the same protein, indicating that the local environment of a single chromophore within a single binding site may differ in a manner dependent upon other molecular characteristics of the hapten. When a given hapten was associated with the MOPC-315 and MOPC-460 proteins, protein-dependent differences in the magnitude, the sign and/or the wavelength maxima of individual CD bands were observed. The occurrence of multiple individually varying CD bands in each induced spectrum increases the usefulness of circular dichroism as a probe of binding site fine structure. Jaffe *et al.* (1971) have reported that the MOPC-315 and MOPC-460 proteins differ in their relative affinities for haptens. The present experimental results furnish additional evidence that the combining sites of these two mouse IgA proteins differ in structure. Circular dichroism and equilibrium dialysis with multiple haptens offer complementary systems for comparative studies of individual combining sites.

Protein-dependent variations in induced circular dichroic bands also were demonstrated with antibodies produced by conventional means, including heterogeneous antibodies elicited against the same haptenic determinant in the same species. The index of heterogeneity (a) values for the goat anti-Dnp preparations were substantially less than unity, indicating that each was composed of antibodies of widely varying affinity. This association constant heterogeneity was reflected in the circular dichroism data. The CD spectra generated by complexing Dnp-lysine with two anti-Dnp preparations of higher and lower affinity separated from a single bleeding of a single goat varied in the magnitude and λ_{max} of CD bands and in crossover points, demonstrating that different antibodies in a heterogeneous population will make separate and distinct contributions to the sum of the induced circular dichroism of the total population. Antibody preparations with association constant heterogeneity have been examined in hapten excess where the CD spectrum is the sum of the induced circular dichroism of all individual antibody–hapten complexes. The construction of CD hapten binding curves furnishes additional data for assessment of antibody combining site heterogeneity. During the sequential addition of hapten, antibody association constant heterogeneity results in a changing proportion of higher and lower affinity combining site occupied by hapten and changing individual contributions to the total CD spectrum. This was manifested with antibodies of the present study as changes in the ratios of ellipticities at different wavelengths, changes in λ_{max} values of CD bands, and, with spectra displaying crossover points, changes in the wavelength of crossing-over. Similar observations have been made with sheep anti-Dnp antibodies (K. J. Dorrington and P. M. Holland, unpublished observation). These findings are to be contrasted with the invariant ellipticity ratios, λ_{max} values and isosbestic crossover points observed with the homogeneous MOPC-315 and MOPC-460 proteins. The importance of using homogeneous immunoglobulins when specific CD spectral details are to be considered is obvious.

Reid *et al.* (1971) have recently examined the circular dichroism of a rabbit anti-Dnp antibody–Dnp-lysine complex and a mouse anti-Tnp antibody–Tnp-lysine complex between 300 and 540 nm and have observed extrinsic Cotton effects centered at transitions of the Dnp and Tnp groups. The circular dichroic spectra obtained with these heterogeneous anti-Dnp and anti-Tnp antibodies, examined in the region of antibody excess (Reid *et al.*, 1971), differ strikingly from spectra reported in the present communications. Extrinsic Cotton effects occurring when *p*-azobenzenearsonate antigen is bound by specific antibody also have been reported (Conway-Jacobs *et al.*, 1970). In addition, extrinsic Cotton effects have been observed when the hapten *p*-(*p*-dimethylaminobenzeneazo)phenyl β-lactoside is bound by specific antibody (J. H. Rockey, unpublished observation).

The circular dichroic spectra obtained with the three goat anti-Dnp antibody preparations resembled each other more closely than they do spectra generated with proteins from other species. The amino-terminal sequences of heavy and light chains display sets of species-specific amino acid resi-

dues as well as hypervariable regions (Milstein and Munro, 1970; Press and Hogg, 1970; Pink *et al.*, 1971) and the possibility that species-specific residues may contribute to antibody combining site structure is to be considered. The present experimental results suggest that circular dichroism may furnish a useful parameter for evaluating species-specific features of binding site structure.

Temperature-dependent changes in the magnitude and λ_{max} of CD bands provide a measure of the flexibility of localized structural features of macromolecules (Fretto and Strickland, 1971a). The stability of the details of the induced circular dichroism of high-affinity complexes of MOPC-315 and MOPC-460 proteins and haptens between 4 and 37° indicates that the ligand chromophores and combining site residues are maintained in a highly preferred stereochemical relationship. This interpretation is consistent with the observations of Hamovich *et al.* (1970) that two affinity labeling reagents, bromoacetyl-*N*-Dnp-ethylenediamine and bromoacetyl-N^{ϵ}-Dnp-lysine which differ in the separation of the Dnp- and the reactive bromoacetyl groups, couple wholly with either a light-chain tyrosyl or a heavy-chain lysyl residue, respectively, of the MOPC-315 protein.

The large negative CD band in the spectra of the Dnp- and Tnp-hapten–MOPC-315 protein complexes, centered in the protein aromatic residue absorption region near 300 ± 15 nm where haptens display absorption minima, may in part represent induced optical activity of a protein binding site chromophore (*e.g.*, tryptophanyl and/or tyrosinyl residue) generated by an intermolecular coupled oscillator mechanism (Woody and Tinoco, 1967; Eyring *et al.*, 1968; Höhn and Weigang, 1968; Hooker and Schellman, 1970; Philipson *et al.*, 1971; Atkins, 1971; Hsu and Woody, 1971; Fretto and Strickland, 1971b; Kägi *et al.*, 1971). An apparent reciprocal relationship (Eyring *et al.*, 1968; Urry, 1970) occurs in the induced CD spectra of Dnp-aminocaproate, Dnp-glycine, Dnp-glycine methyl ester, and Tnp-aminocaproate complexed with MOPC-315 protein, between the positive 370- to 382-nm CD band of the haptenic chromophore and the negative (300 ± 15)-nm CD band. While the magnitudes of the rotational strengths of these bands vary widely between the different Dnp- and Tnp-hapten–MOPC-315 protein spectra, the ratio of the rotational strength of the 370- to 382-nm CD band to that of the (300 ± 15)-nm CD band remains largely unchanged. Methylation of the α-carboxyl group of Dnp-glycine altered the circular dichroic spectrum for this bound hapten. Coulombic interactions between a negatively charged α-carboxyl group of Dnp-glycine and charged groups of the antibody combining site may lead to a stereochemical orientation of the Dnp group and a protein chromophore that differed from that obtained with the Dnp-glycine methyl ester. Alternately, the negatively charged vicinal α-carboxyl group of the bound Dnp-glycine may perturb electronic transitions of the Dnp chromophore and of an antibody site chromophore in a reciprocal manner (*cf.* Hooker and Schellman, 1970; Urry, 1970). It is of interest to note that the presence or absence of an α-amino group does not influence the induced circular dichroic spectrum of the N^{ϵ}-Dnp group, obtained by binding either Dnp-lysine or Dnp-aminocaproate to the same homogeneous MOPC-315 protein. The observed quenching of immunoglobulin tryptophan fluorescence by bound haptens and the occurrence of a shift of the 293-nm CD band of the MOPC-315 protein spectrum to 292 nm in the Dnp-aminocaproate–MOPC-315 protein spectrum (J. H. Rockey and R. M. Freed, unpublished observation) (*cf.* Fretto and Strickland, 1971a,b; Barth *et al.*, 1971; Wollmer and Buse, 1971) argue for a tryptophanyl residue participating in intermolecular dynamic coupling (Eyring *et al.*, 1968). Circular dichroism studies at ultra-low temperatures (*cf.* Fretto and Strickland, 1971a,b) and chemical modification of tryptophanyl and tyrosinyl residues may be of use in defining the role of these residues in generating optical activity of hapten–antibody complexes, and such studies now are in progress in our laboratory. Studies with model compounds also may be of use in defining interactions occurring between Dnp and Tnp groups and the indole moiety. L-Tryptophan displays positive CD bands at 291, 281, and 272 nm (Myer and MacDonald, 1967) whereas N^{α}-Dnp-L-tryptophan has negative CD bands in in this spectral region (J. H. Rockey and K. J. Dorrington, unpublished observation).

The affinity of a protein may vary substantially for two haptens that possess the same chromophore but differ in other moieties (*e.g.*, Dnp-aminocaproate and Dnp-glycine methyl ester). The observation that induced CD bands centered at ligand chromophore transitions differ for the two bound haptens indicates that interactions occurring between the single chromophoric moiety and protein residues differ for the two ligands. An altered free energy change resulting from an altered stereochemical orientation of the common chromophoric moiety within the binding site, as well as additional interactions occurring between other ligand moieties and the protein, both may contribute to the affinity differences.

The sensitivity of the details of the induced circular dichroic spectra to small variations in antibody combining site structure makes analysis of optical activity of antibody–hapten complexes a powerful method with which to study and compare the fine structure of the combining sites of antibodies of related specificity, to study the association of haptens with isolated antibody subunits, to probe the re-formed combining site resulting from the reassociation of antibody heavy and light polypeptide chains, and to assay the stability of combining sites under different conditions of solvent and temperature.

Acknowledgments

We thank Doctor Robert C. Davis of the Department of Chemistry, University of Pennsylvania, for the use of his calibrated Jasco-Durrum Model J-10 spectropolarimeter, and Miss Ada C. Bello for excellent technical assistance. We also thank Doctor Herman N. Eisen for the mouse tumor cell lines.

Addendum

Additional studies of the extrinsic Cotton effects of antibody–hapten complexes have been recently reported by Glaser and Singer (1971).

References

Atkins, P. W. (1971), *Chem. Brit. 7*, 244.

Barth, G., Records, R., Bunnenberg, E., Djerassi, C., and Voelter, W. (1971), *J. Amer. Chem. Soc. 93*, 2545.

Beychok, S. (1968), *Annu. Rev. Biochem. 37*, 437.

Blout, E. R. (1964), *Biopolym. Symp. 1*, 397.

Cassim, J. Y., and Yang, J. T. (1969), *Biochemistry 8*, 1947.

Conway-Jacobs, A., Schechter, B., and Sela, M. (1970), *Biochemistry 9*, 4870.

Dammkoehler, R. A., and Gallagher, T. L. (1971), *Methods Immunol. 3*, 407.
Eisen, H. N., Gray, W. G., Little, J. R., and Simms, E. S. (1967), *Methods Immunol. 1*, 351.
Eisen, H. N., and McGuigan, J. E. (1971), *Methods Immunol. 3*, 395.
Eisen, H. N., Simms, E. S., and Potter, M. (1968), *Biochemistry 7*, 4126.
Eyring, H., Liu, H.-C., and Caldwell, D. (1968), *Chem. Rev. 68*, 525.
Fretto, L., and Strickland, E. H. (1971a), *Biochim. Biophys. Acta 235*, 473.
Fretto, L., and Strickland, E. H. (1971b), *Biochim. Biophys. Acta 235*, 489.
Glaser, M., and Singer, S. J. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2477.
Haimovich, J., Givol, D., and Eisen, H. N. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1656.
Höhn, E. G., and Weigang, O. E. (1968), *J. Chem. Phys. 48*, 1127.
Hooker, T. M., Jr., and Schellman, J. A. (1970), *Biopolymers 9*, 1319.
Hsu, M.-C., and Woody, R. W. (1971), *J. Amer. Chem. Soc. 93*, 3515.
Jaffe, B. M., Simms, E. S., and Eisen, H. N. (1971), *Biochemistry 10*, 1693.
Kägi, J. H. R., Li, T.-K., and Vallee, B. L. (1971), *Biochemistry 10*, 1007.
Milstein, C., and Munro, A. J. (1970), *Annu. Rev. Microbiol. 24*, 335.
Moscowitz, A. (1960), *in* Optical Rotatory Dispersion, Djerassi, C., Ed., New York, N. Y., McGraw-Hill, p 150.
Myer, Y. P., and MacDonald, L. H. (1967), *J. Amer. Chem. Soc. 89*, 7142.
Nakazawa, A., Nakazawa, T., Kotani, S., Nozaki, M., and Hayaishi, O. (1969), *J. Biol. Chem. 244*, 1527.
Perrin, J. H., and Hart, P. A. (1970), *J. Pharmaceut. Sci. 59*, 431.
Philipson, K. D., Tsai, S. C., and Sauer, K. (1971), *J. Phys. Chem. 75*, 1440.
Pink, R., Wang, A.-C., and Fudenberg, H. H. (1971), *Annu. Rev. Med. 22*, 145.
Porath, J., Axen, R., and Ernback, S. (1967), *Nature (London) 215*, 1491.
Potter, M., and Lieberman, R. (1967), *Advan. Immunol. 7*, 91.
Press, E. M., and Hogg, N. M. (1970), *Biochem. J. 117*, 641.
Reid, V., Glaser, M., Kennett, R., and Singer, S. J. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1184.
Rockey, J. H. (1967), *J. Exp. Med. 125*, 249.
Rockey, J. H., Dorrington, K. J., and Montgomery, P. C. (1971a), *Nature (London) 232*, 192.
Rockey, J. H., Dorrington, K. J., and Montgomery, P. C. (1971b), *J. Immunol. Methods 1*, 67.
Stevenson, G. T. (1968), *Bibl. Haematol. 29*, 537.
Stevenson, G. T., and Dorrington, K. J. (1970), *Biochem. J. 118*, 703.
Tinoco, I., and Cantor, C. R. (1970), *Methods Biochem. Anal. 18*, 81.
Ulmer, D. D., and Vallee, B. L. (1965), *Advan. Enzymol. 27*, 37.
Underdown, B. J., Simms, E. S., and Eisen, H. N. (1971), *Biochemistry 10*, 4359.
Urry, D. W. (1970), *in* Spectroscopic Approaches to Biomolecular Conformation, Urry, D. W., Ed., Chicago, Ill., American Medical Association, p 33.
Urry, D. W., and Pettegrew, J. M. (1967), *J. Amer. Chem. Soc. 89*, 5276.
Velick, S. F., Parker, C. W., and Eisen, H. N. (1960), *Proc. Nat. Acad. Sci. U. S. 46*, 1470.
Wollmer, A., and Buse, G. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 16*, 307.
Woody, R. W., and Tinoco, I. (1967), *J. Chem. Phys. 46*, 4927.
Wu, W.-H., and Rockey, J. H. (1969), *Biochemistry 8*, 2719.