Nuclear Magnetic Resonance and Fluorescence Studies of the Binding of *O*-Carboxymethyl-4-methylumbelliferone to Its Specific Antibody†

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ABSTRACT: The interaction of a new type of hapten, *O*-carboxymethyl-4-methylumbelliferone (CMU), with its specific IgG antibody and with the Fab fragment of the antibody was studied by 250-MHz proton nuclear magnetic resonance (NMR) and by fluorescence spectroscopy. The hapten fluorescence was quenched by either the IgG or the Fab and by dioxane–water mixtures, and these findings suggest a hydrophobic environment in the active site. Further support for this argument was provided by the positive temperature dependence of the binding constant for the hapten–antibody interaction. There was little quenching of the tryptophan fluorescence of the antibody, however, despite the considerable overlap between the emission spectrum of the antibody and the excitation spectrum of the hapten. This observation suggests that there are no tryptophan residues in the combining site. The NMR studies indicated that there were no tryptophan or other aromatic residues in the antibody binding site since no significant chemical shift of the CMU proton resonance signals was observed on binding. CMU bound to the antibody or to the Fab fragment showed differentially broadened proton resonances and exhibited differential nuclear Overhauser effects. The relaxation of the H_5 and H_6 protons on the hapten was dominated by intramolecular magnetic dipole interaction, while the other protons on the hapten were relaxed primarily by magnetic dipole interaction with protons in the binding site of the antibody. The data led to a rather detailed model of the antibody combining site and the way in which the hapten was bound. The site appears to be a crevice in which the hapten is oriented such that its lactone ring is innermost.

A number of chemical and physical methods for examining the structure and properties of the antibody binding site have been devised. Fluorescence has provided information regarding the dielectric nature of the binding site, the conformational changes resulting from complex formation, and the strength of the binding interaction (Parker et al., 1967a,b; Werner et al., 1972; Eisen and Siskind, 1964). Nuclear magnetic resonance (NMR) has been useful in studying the binding of ligands to proteins (Dwek, 1973; James, 1975), and the intermolecular nuclear Overhauser effect[1] has yielded information on the chemical nature of the groups in the binding sites and their distances from the bound molecule (Bothner-By and Gassend, 1973; Balaram et al., 1972a,b, 1973; James, 1976). several studies have utilized conventional NMR techniques to investigate the antibody–hapten interaction: Haughland et al. (1967) used the chloride ion probe to study the binding of a dinitrophenylmercurial hapten with a DNP-specific antibody; Burgen et al. (1967) investigated the binding of tetramethylammonium ions to IgG antibody specific for phenoxycholine; and Dwek et al. (1975a,b) reported studies on the binding site of the myeloma protein MOPC-315 in which they suggested that the combining site contains 30 aromatic and 30 aliphatic protons.

The purpose of this study was to develop a hapten that would be useful for probing the structure of the antibody combining site and then to use it to develop a model of the combining site and the way in which the hapten is bound. *O*-Carboxymethyl-4-methylumbelliferone was chosen because it fluoresces strongly, gives a clear nuclear magnetic resonance spectrum, and contains no nitrogen or other quadrupolar nuclei which could complicate the interpretation of the nuclear spin relaxation.

Materials and Methods

Preparation of the Antigens. *O*-Carboxymethyl-4-methylumbelliferone (CMU). CMU was synthesized by the reaction of chloroacetic acid with 4-methylumbelliferone (MU) in the presence of base: 26.4 g (0.15 mol) of MU and 28.2 g (0.3 mol) of Cl_3CCOOH were reacted in a solution of 21.6 g of NaOH in 300 mL of H_2O under reflux for 4 h, then cooled in an ice–water bath, and acidified with 47 mL of concentrated HCl. The precipitate was collected and dissolved in a solution of 15 g of $NaHCO_3$ in 300 mL of H_2O. Unreacted and undissolved MU was removed by filtration, and the filtrate was collected and acidified to pH 2 to precipitate the CMU product. The CMU was recrystallized from 600 mL of an ethanol–water mixture (50:50 v/v) to yield 25.4 g (75%) of CMU melting at 206–207 °C. The elemental analysis (Geller Microanalytical Laboratories, Saddle River, N.J. 07458) was carbon 61.52%, hydrogen 4.35%, and oxygen 34.10%, and the theoretical values are carbon 61.54%, hydrogen 4.30%, and oxygen 34.16%.

CMU–Acid Chloride. In order to activate CMU for coupling with a protein carrier, it was converted to the acid chloride by reaction with PCl_5: 5 g (0.02 mol) of CMU and 6.5 g (10% excess) of PCl_5 were reacted in 100 mL of dry dioxane

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[1] Abbreviations used: NOE, nuclear Overhauser effect; CMU, *O*-carboxymethyl-4-methylumbelliferone; MU, 4-methylumbelliferone; PBS, phosphate-buffered NaCl (0.01 M phosphate–0.14 M NaCl, pH 7.6); TSP-d_4, deuterated sodium 3-trimethylsilyl propionate; THP, terepthalic acid; BGG, bovine γ-globulin.

under reflux for 4 h. The volume was reduced to half by distillation, activated charcoal was added, and the solution was filtered while hot. The acid chloride was precipitated from the filtrate by the addition of 100 mL of hexane. The solvent and the by-product $POCl_3$ were removed under vacuum.

O-Carbobenzoxy-L-tyrosine. *O*-Cbz-L-tyrosine was synthesized by the method of Overell and Petrow (1955). The product was recrystallized from 20% acetic acid in water (v/v), and it melted at 215 °C with decomposition, in agreement with the literature value (Katchalski and Sela, 1953).

N-Carboxy Anhydride (NCA) of *O*-Cbz-L-tyrosine, ε-Cbz-L-lysine, and γ-Benzyl-L-glutamate. The conversion of the blocked amino acids into the *N*-carboxy anhydrides was done by the phosgenation procedure of Fasman et al. (1961). From 5 g of the blocked amino acids, *O*-Cbz-L-tyrosine gave an 83% yield of the NCA (mp 100–101 °C dec), ε-Cbz-L-lysine gave an 86% yield (mp 99–100 °C dec), and γ-benzyl-L-glutamate gave a 92% yield (mp 94–95 °C dec).

The Carriers. Poly($Glu^{52}Lys^{33}Tyr^{15}$) (mol wt 27 000) and poly($Glu^{60}Lys^{40}$) (mol wt 82 000) were synthesized by copolymerization of the NCAs (Friedman et al., 1962). Bovine γ-globulin (BGG) was purchased from Pentex Inc. (Kankakee, Ill.).

Coupling of the CMU Hapten to the Carriers. The CMU-acid chloride was coupled to each of the three carriers. The carrier (500 mg) was dissolved in 85 mL of 0.05 M carbonate-bicarbonate buffer, pH 10.5, and the solution was cooled in an ice-water bath; when necessary the pH was adjusted to 10.3–10.5 with 1 N NaOH added dropwise. The CMU-acid chloride (200 mg) was added over the period of an hour, and when the pH did not change appreciably from 10.5 for about 30 min, the reaction was allowed to proceed at room temperature for 20 h. The solution was filtered through a coarse sintered disc funnel and dialyzed twice in 0.15 M PBS (phosphate-buffered NaCl: 0.01 M phosphate–0.14 M NaCl, pH 7.6) to remove the CMU which formed as the hydrolysis product of the acid chloride. The dialyzate was passed through Sephadex G-25 (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) in a column (45 × 3 cm) of 500 mL total volume using the same buffer. The eluate was monitored at the hapten absorbance of 320 nm. The fraction corresponding to the hapten-coupled carrier was dialyzed three times in distilled H_2O and then lyophilized.

Immunization. New Zealand white rabbits were immunized with either 2 mg or 5 mg of the CMU-carrier complex dissolved in 0.1 mL of PBS and emulsified with an equal volume of complete Freund's adjuvant. The emulsion was injected subcutaneously into each hind foot pad (0.3 mL) and the back of the neck (0.4 mL). Three weeks later, the rabbits were test bled and injected with the same amount of antigen dissolved in 1 mL of PBS: 0.3 mL into each thigh and 0.4 mL into the back of the neck. The rabbits were bled 7 days later by cardiac puncture.

Isolation and Purification of the Antibody. The CMU was coupled to AH-Sepharose 4B to form an affinity column. The Sepharose (2.1 g) was swollen in an excess of 0.5 M NaCl and washed with distilled water. The CMU (60 mg) was dissolved in 15 mL of 0.05 M $NaHCO_3$, and the pH was adjusted to 6. The Sepharose gel was added to the hapten solution, and the volume was adjusted to make a slurry of sufficient viscosity for stirring. Then 700 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride was added in small portions to the slurry, and the pH was maintained between 4.5 and 6.0. When the changes in pH were small, the reaction was allowed to proceed at room temperature for 24 h. The affinity gel was washed thoroughly with PBS using a coarse sintered disc funnel, made into a slurry, and poured into a column (0.9 × 30 cm).

Isolation of CMU Specific Antibodies by Affinity Chromatography. The γ-globulin fraction (70 mL), which was prepared by ammonium sulfate precipitation at 50% saturation, was passed through the affinity column in distilled H_2O at the rate of 0.3 mL/min and monitored at 278 nm. The column was washed until 350 mL of buffer showing no absorbance was obtained. The CMU-specific antibody was eluted completely with 0.1 M acetic acid–0.05 M NaCl, and 90% of it was eluted with 0.01 M acetic acid–0.14 M NaCl. High salt concentrations (3 M NaCl) did not dissociate the antibody. The fractions were dialyzed three times against PBS and passed through a column of AH-Sepharose 4B to remove any proteins that might have bound nonspecifically to the affinity column. The purified antibody was fully active and specific for CMU since it bound completely to the CMU affinity column when it was repassed through it. The antibody was stored frozen at −20 °C until used in the NMR, fluorescence and equilibrium dialysis experiments.

Preparation of the Fab Fragment. The method of Porter (1959) was used: 70 μL of Hg papain (Worthington Biochemical Corp., Freehold, N.J.) (271 units/mL; 15.4 mg/mL) was added to a solution of 30 mg of purified antibody in 15 mL of buffer (0.1 M sodium phosphate–0.01 M cysteine–2 mM ethylenediaminetetraacetate, pH 7.0), and a few drops of toluene was added as an antifoaming agent. The reaction proceeded for 18 h at 22 °C, and then the solution was dialyzed three times against PBS and passed through the affinity column to isolate the Fab fragments.

Precipitin Assay. These assays employed 0.25 mL of varying concentrations of antigen in PBS and the same volume of the antiserum (Gill, 1967). The reaction mixture was incubated for 3 days at 4 °C. The precipitate was washed with PBS and dissolved in 0.5 mL of 0.25 N acetic acid; the absorbance at 278 nm and 320 nm was measured. The absorbance at 278 nm was converted to antibody concentration, after correcting for the amount of antigen present, by using the extinction coefficient of 1.5 mL mg^{-1} cm^{-1}. Since the antigen contains CMU which absorbs at 320 nm, the correction for the contribution of the antigen was made by multiplying the reading at 320 nm by the predetermined ratio of the absorbance at 278 nm to the absorbance at 320 nm for the CMU-carrier complex and subtracting this from the absorbance measured at 278 nm to obtain the absorbance of the antibody alone.

Equilibrium Dialysis. Carbon-14 labeled CMU ([^{14}C]-CMU) was synthesized by reacting $ClCH_2{}^{14}COOH$ (0.4 mg, 250 μCi; Amersham/Searle Corp., Arlington Heights, Ill.) dissolved in 1 mL of H_2O with MU (25 mg) dissolved in 1.5 mL of 0.1 N NaOH. The mixture was reacted under reflux for 12 h, and then excess MU was precipitated by adjusting the pH to 7. After filtering, the filtrate was passed through an anion-exchange column (0.9 × 30 cm; Bio-Rex 5, chloride form, 200–400 mesh; Bio-Rad Laboratories, Richmond, Calif.). Unreacted MU was eluted with 0.2 M acetate buffer at pH 8, and [^{14}C]CMU was eluted with the same buffer at pH 4. Since CMU fluoresces at 387 nm while MU fluoresces at 445 nm, the separation was monitored by fluorimetry. The [^{14}C]CMU was not contaminated with any unreacted $ClCH_2{}^{14}COOH$, which had been added in stoichiometric amounts, since it would not eluate unless the pH was much lower. Equilibrium dialysis of the purified antibody with the ^{14}C-labeled hapten was performed in 1-mL cells for 24 h, as described previously (Ruscetti et al., 1974).

Fluorescence. The fluorescence measurements were made on a Farrand MK-1 fluorimeter at 22 °C. The fluorescence

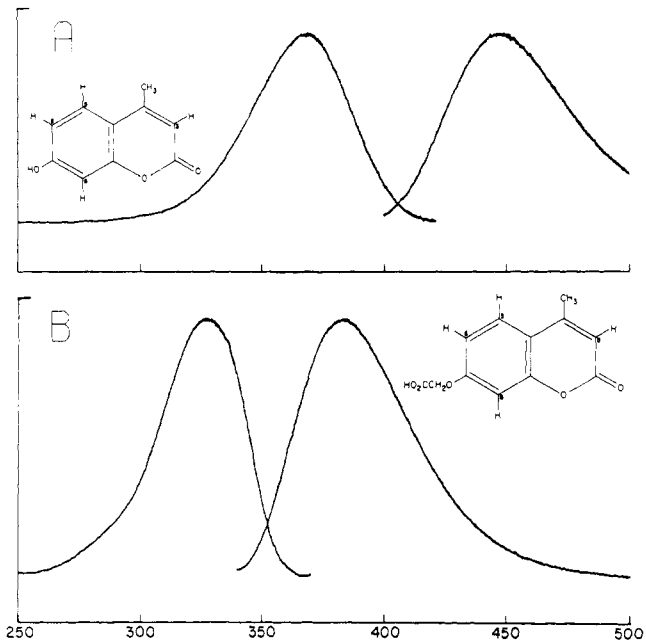

FIGURE 1: (A) Fluorescence spectrum of 1.0×10^{-6} M 4-methylumbelliferone (MU) in 0.2 M Na_2CO_3, pH 10.4. (B) Fluorescence spectrum of 1.0×10^{-6} M *O*-carboxymethyl-4-methylumbelliferone (CMU) in PBS, pH 7.6. The scales are in nanometers.

titrations were performed directly in a quartz cuvette (4 × 4 × 9 cm) provided with a micromagnetic stirrer and containing 2 mL of the antibody or the hapten in 0.15 M PBS. The titrant was added in 10–30-μL aliquots, and the titration curves were corrected for volume changes. A buffer blank was used for zeroing, and control titrations were done simultaneously. The light scattering at 360 nm (excitation at 315 nm) of a Pyrex glass standard (Precision Cells Inc., Hichsville, N.Y.) was used to correct for sensitivity drifts in the detection system by adjusting the gain. Drifts are minimal within the 2-h measurement period.

Nuclear Magnetic Resonance. The proton NMR spectra were taken on a 250-MHz spectrometer of the NMR Facility for Biomedical Studies at Carnegie-Mellon University. The spectrometer, operating in a frequency sweep mode, has an XDS-Sigma 5 computer interface for real-time signal accumulation. Each spectrum represents 4096 time-averaged points analyzed by cross-correlation with a calculated reference line. Double resonance for the nuclear Overhauser effect (NOE) experiments was performed by irradiating at a fixed point in the aliphatic region of the antibody using a second frequency (H_2), while the H_1 frequency was in the sweep mode in the aromatic region. The difference spectra were obtained by digital subtraction of the "on resonance" spectra from the "off resonance" spectra under the same operating conditions. "Off resonance" refers to a condition in which the saturating (H_2) frequency is set away from, or off, the resonances of the antibody. "On resonance" is the condition in which the irradiating frequency is right on the resonance under study. Trifluoroacetic acid in an external capillary was used as the external proton lock. TSP-d_4 (deuterated sodium 3-trimethylsilyl propionate) was used as the internal aliphatic reference standard, and terephthalic acid was used as the internal aromatic reference standard. All solutions were prepared in 0.15 M PBS in D_2O, pD 7.6.

Results

Antibody Responses to the CMU-Carrier Conjugates. Bovine γ-globulin, poly($Glu^{60}Lys^{40}$) and poly(Glu^{52}-$Lys^{33}Tyr^{15}$) were investigated for their suitability as carriers for *O*-carboxymethyl-4-methylumbelliferone (molar hapten–carrier ratios of 6, 16, and 9, respectively). The antibody responses against these antigens decreased in the order: CMU-BGG (1500–1800 μg of Ab/mL) > CMU-poly-($Glu^{52}Lys^{33}Tyr^{15}$) (250–550 μg) > CMU-poly($Glu^{60}Lys^{40}$) (50–330 μg). About 95% of the total anti-CMU-BGG antibody and 55% of the total anti-CMU-poly($Glu^{52}Lys^{33}Tyr^{15}$) antibody were directed specifically against the hapten. The antibody-CMU-BGG complex was completely dissociated by 10^{-2} M CMU in PBS, pH 7.6, and this finding supports the conclusion that the antibody is hapten specific. A solution of 0.1 M acetic acid–0.05 M NaCl, which completely dissociated the complex, was used routinely for eluting antibodies from the affinity column. Anti-CMU antibody elicited by immunization with two doses of 5 mg/mL CMU-BGG was used in all of the studies reported here.

Fluorescence Studies. The fluorescence spectra of 4-methylumbelliferone and its carboxymethyl derivative (CMU) are shown in Figure 1. There is a blue shift in the fluorescence maximum from 449 nm in the parent compound to 387 nm in the derivative, and a smaller blue shift in the excitation maximum from 367 nm in the parent compound to 327 nm in the derivative. The molar extinction coefficient of CMU at 320 nm is 14 000; Sherman and Robins (1968) reported the value of 17 000 for MU.

The quenching of CMU fluorescence by the purified, hapten-specific IgG antibody is shown in Figure 2A. Control titrations using the nonbinding γ-globulin fraction that passed through the affinity column and normal rabbit γ-globulin did not cause any quenching of the hapten fluorescence. The hapten fluorescence is progressively quenched in dioxane–water mixtures, and the quenching exceeds 95% in pure dioxane.

The binding constant for the hapten–antibody interaction was measured from the quenching of hapten fluorescence rather than from the quenching of the antibody tryptophan fluorescence (Eisen and Siskind, 1964), since the maximal quenching of the tryptophan in the CMU-anti-CMU antibody system was only 10%. The maximal quenching of the hapten, Q_{max}, was obtained from the titration curve in Figure 2A by extrapolating the hapten–antibody ratio to zero. From the value of the quenching at the ith titration point, Q_i, the value of r (mol of hapten bound/mol of antibody) in a Scatchard calculation (Scatchard, 1949) can be estimated from $2[1 - (Q_i/Q_{max})]$; the value of 2, which was obtained from the convex curve in Figure 2A (see below), was used as the number of binding sites in the IgG antibody. The concentration of free hapten, c, was calculated from $[1 - (Q_i/Q_{max})]H$, where H is the concentration of the total hapten added. Thus, the concave quenching curve in Figure 2A can be translated into the Scatchard plot in Figure 2B. The nonlinear plot suggests heterogeneity in the binding constants of the different antibody populations. The index of heterogeneity, a, as well as the average binding constant, K, may be obtained by transforming the nonlinear Scatchard plot into the linear Sips function (Sips, 1948) of eq 1. This graph is shown in Figure 2C.

$$\log(r/(N - r)) = a \log K + \log c \qquad (1)$$

The convex curve in Figure 2A is the change in hapten fluorescence per unit hapten added and reflects the rate of change of the hapten fluorescence as the antibody is progressively titrated with the hapten. The fluorescence per unit of free hapten in buffer is a constant value which is the limit being approached by the progressive titration of the antibody. The number of

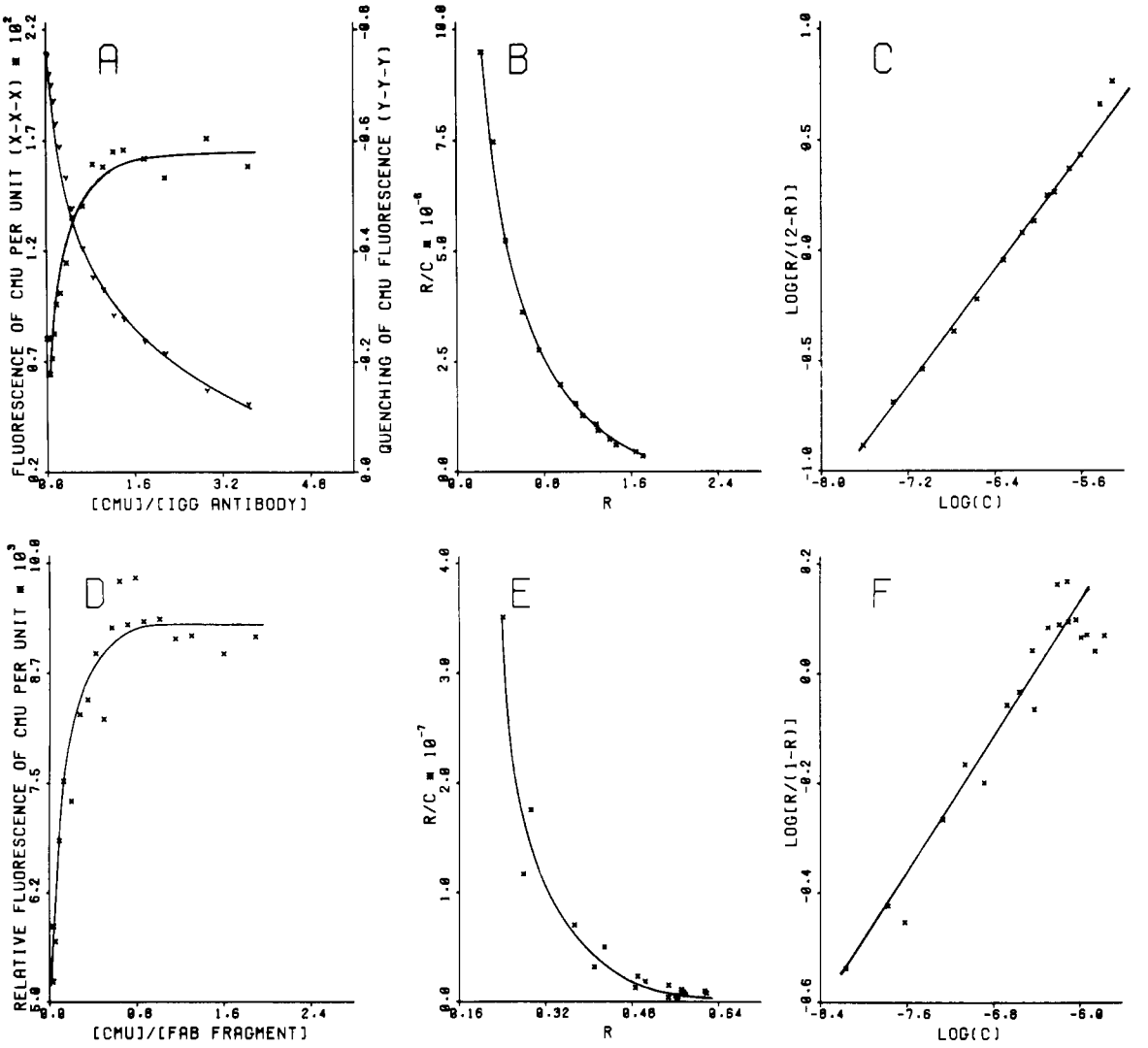

FIGURE 2: (A) Titration of 1.6×10^{-6} M IgG antibody with CMU in PBS, pH 7.6; quenching of hapten fluorescence (Y-Y-Y) relative to the titration of a control buffer and change in hapten fluorescence per unit hapten added (X-X-X). (B) The Scatchard plot of A. (C) The Sips plot of A. (D) Fluorescence titration of 1.6×10^{-6} M Fab with CMU in PBS, pH 7.6. (E) The Scatchard plot of D. (F) The Sips plot of D.

TABLE I: Binding Parameters of CMU to its Specific Antibody.

Purified IgG antibody preparation	Average binding constant from Sips transform of quenching curve (10^{-6} L/mol)	Binding constant from equilibrium dialysis (10^{-6} L/mol)	Hetero-geneity index	Q_{max}
6	1.9		0.70	0.74
7	1.3	2.5	0.63	0.94
8	2.2	2.6	0.78	0.84
Mean	1.8	2.6	0.70	0.84

binding sites (2) may then be determined from the beginning of the plateau of the curve, which is the point where all of the binding sites have reacted with the hapten.

Figures 2D-2F are the same titration plots for the Fab fragment. In this case the number of binding sites is 1. The binding constant of the Fab is 1.4×10^6 which is approximately the same as the average value of 1.8×10^6 for the IgG antibody. The binding constants obtained from fluorescence titration agree with those obtained from equilibrium dialysis (Table I).

Thermodynamic Studies. The enthalpy change associated with the antibody-hapten reaction was measured by plotting the binding constants obtained from equilibrium dialysis at three different temperatures (4, 22, 37 °C) as an Arrhenius plot. The calculated thermodynamic values at 298 K are: ΔH = 5100 cal/mol, ΔF = −8800 cal/mol, and ΔS = 47 cal deg^{-1} mol^{-1}.

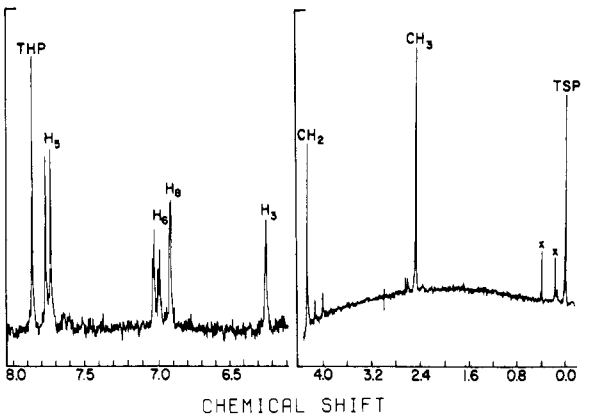

FIGURE 3: The 250-MHz NMR spectra of 1 mM CMU in deuterated PBS, pD 7.6. HDO solvent impurities are marked X. THP is terephthalic acid.

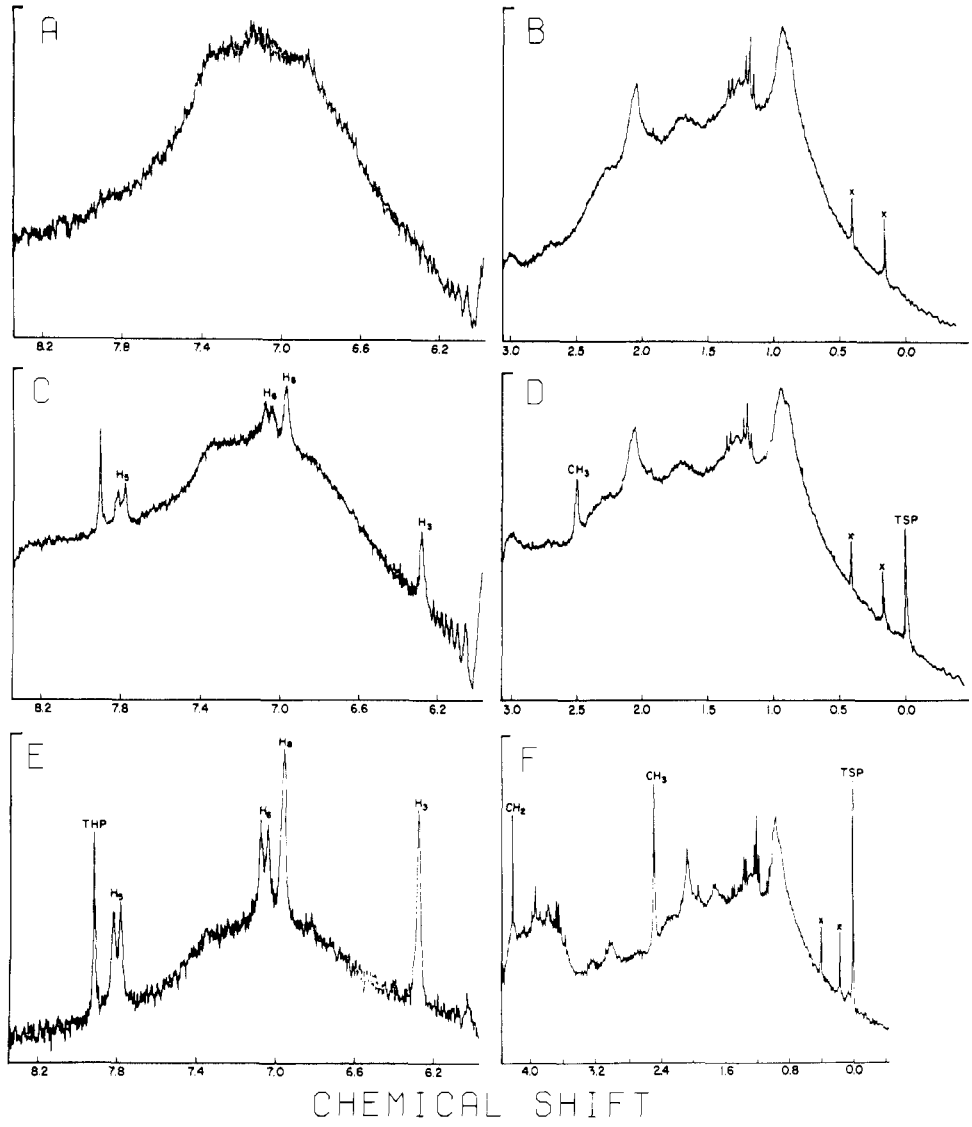

FIGURE 4: (A, B) The NMR spectra of 1.2×10^{-5} M IgG antibody in deuterated PBS, pD 7.6. (C, D) The NMR spectra of 1.2×10^{-5} M IgG antibody + 0.3 mM CMU. (E, F) The NMR spectra of 1.2×10^{-5} M IgG antibody + 1 mM CMU.

TABLE II: NMR Parameters for CMU in Phosphate-Buffered Sodium Chloride.[a]

Protons on CMU	Chemical shift (ppm)
CH_3	2.47
CH_2	4.28
H_3	6.25
H_8	6.93
H_6	7.02
H_5	7.75
Terephthalic acid	7.87
TSP[b]	0.00

Coupling values (Hz): $J_{6,8} = 2.2$; $J_{5,6} = 9.3$

[a] 1 mM CMU in 0.15 M PBS in D_2O, pD 7.6. [b] Internal reference.

TABLE III: Line Broadening of CMU by Specifically Purified IgG Antibody and Fab.[a]

Solution	Line width (Hz)			
	CH_3[b]	CH_2	H_3[b]	H_8[b]
1 mM CMU	3.1	2.6	3.5	3.6
1 mM CMU + 1.2×10^{-5} M IgG	5.2	2.6	4.5	5.3
1 mM CMU + 1.1×10^{-4} M Fab	6.5	2.9	4.8	5.5
Broadening due to IgG	2.1	0.0	1.0	1.7
Broadening due to Fab	3.4	0.3	1.3	1.9

[a] In 0.15 M PBS in D_2O, pD 7.6. Slight adjustments were made for instrumental inhomogeneity contributions as determined from the internal reference standards. [b] Coupling values included.

NMR Line Broadening. The 250-MHz NMR proton line assignments for the CMU hapten in 0.15 M PBS in D_2O at pD 7.6 were made by first-order analysis. The CMU spectrum is shown in Figure 3, and the chemical shifts and coupling values are summarized in Table II.

The NMR spectra of the specific IgG antibody shown in Figures 4A and 4B lack detail, especially in the aromatic region. Nevertheless, the titration of the hapten with the specific antibody produced striking changes in the NMR spectrum of the hapten which reflect the interaction of the hapten and the antibody (Figures 4C–4F). There were no detectable changes in chemical shifts (<0.01 ppm), but the line widths of the CMU protons were broadened (Table III). The line widths for the H_5 and H_6 protons were difficult to measure due to partial

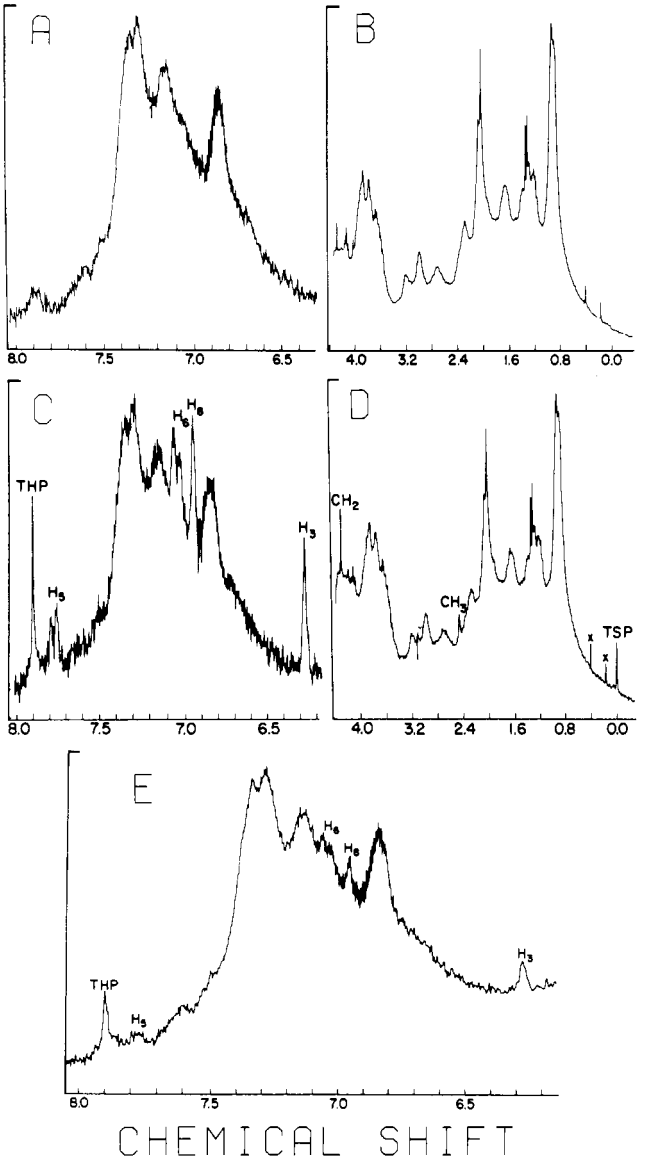

FIGURE 5: (A, B) TheNMR spectra of 1.1×10^{-4} M Fab. (C, D) The NMR spectra of 1.1×10^{-4} M Fab + 1 mM CMU. (E) The aromatic NMR spectrum of 1.1×10^{-4} M Fab + 0.3 mM CMU showing the extensive broadening of the H_5 proton.

coalescence of the multiplets on broadening. Close scrutiny of Figures 3 and 4E reveals that the hapten ring protons are differentially broadened on binding. The H_5 doublet is the peak with highest maximal intensity among the aromatic protons in the absence of the antibody; in the presence of the antibody, the H_5 doublet shows the greatest relative diminution in maximal intensity. On the other hand, the H_3 signal has the greatest maximal intensity in the presence of the antibody. Thus, H_5 gives the most broadened signal, while H_3 gives the least broadened signal. Similarly, comparison of the relative maximal intensities of the H_6 and H_8 proton signals in the absence and in the presence of the antibody shows that the H_6 resonance is the more broadened of the two. Thus, the line broadening of the hapten protons increases in the order $H_3 > H_8 > H_6 > H_5$. The methyl protons are also significantly broadened, but the line widths of the methylene protons are not affected (Figure 4F). Quantitative comparison of peak heights, assuming that the line width at half-height is inversely proportional to the peak height, shows that the ratio of the line width of the proton resonances to the H_3 proton resonance, the least broadened resonance, approaches the limiting values for the hapten in the absence of the antibody (Table IV).

TABLE IV: Peak Height Ratios as a Measure of the Order of Line Broadening of the CMU Protons in the Presence of the Specifically Purified IgG Antibody and Fab.

		H_3/H_5	H_3/H_6	H_3/H_8
		Intensity Ratios		
A.	1 mM CMU	0.64	1.27	0.88
B.	1 mM CMU + 1.2×10^{-5} M IgG	1.82	2.04	1.12
C.	1 mM CMU + 1.1×10^{-4} M Fab	2.18	2.14	1.26
		Percentage		
D.	Deviation of B from A	184	61	27
E.	Deviation of C from A	241	69	43

TABLE V: NMR Parameters for CMU in Various Solvents.

CMU Protons	Solvent: Phosphate buffer, pH 7.6	Dioxane-d_8	Me_2SO-d_6
H_5	5.28	5.21	5.30
H_6	4.55	4.56[a]	5.00[a]
H_8	4.46	4.56[a]	5.00[a]
H_3	3.78	3.79	3.86
CH_2	1.81	2.35	2.42
CH_3[b]	0.00	0.00	0.00

[a] Center of unresolved multiplet. [b] Molecular reference.

Similar results were obtained when the hapten was added to the Fab fragment. The aromatic proton signals of the Fab (Figure 5A) are more resolved than those of the IgG, and the aliphatic region also appears sharper (Figure 5B). The spectra after the addition of 1 mM CMU to the Fab is shown in Figures 5C and 5D: the methyl proton signal is broadened, while the methylene protons are not affected. Analysis of peak heights yields the same order of line broadening of the hapten ring protons as that obtained in the presence of the IgG antibody. The H_5 proton is the most broadened of all resonances, as shown in Figure 5E, where the molar ratio of the hapten to Fab is small. The H_5 proton is hardly visible, whereas, the other proton signals clearly rise out of the baseline noise.

The NMR spectra of CMU were taken in two less polar solvents (dioxane and dimethyl sulfoxide) in order to compare them with the spectra taken in PBS. In order to make the data comparable, the chemical shifts of the CMU protons were measured relative to the methyl group in CMU. The results (Table V) show that the methylene group experiences the greatest shift in the transition from the more polar to the less polar solvents. The H_6 and H_8 protons are moderately shifted, but the H_3 and H_5 protons are not significantly affected. The differential shifts of the CMU protons are directly related to their relative distances from the carboxyl side chains: the protons closer to the acid group are more shifted, and the farther protons are less affected. These results reflect the changes in the ionization state of the carboxyl group which is ionized in the aqueous solution and protonated in the organic solvent. Thus, the observation that no chemical shift occurred when CMU was bound to the hydrophobic pocket of its specific antibody suggests that the carboxyl group is readily accessible to the aqueous environment and that it is in the ionized state in both the free and bound forms.

Nuclear Overhauser Effect. The negative Overhauser effect for the CMU ring protons is shown by the difference spectrum of the "on resonance" and the "off resonance" spectra in Figure

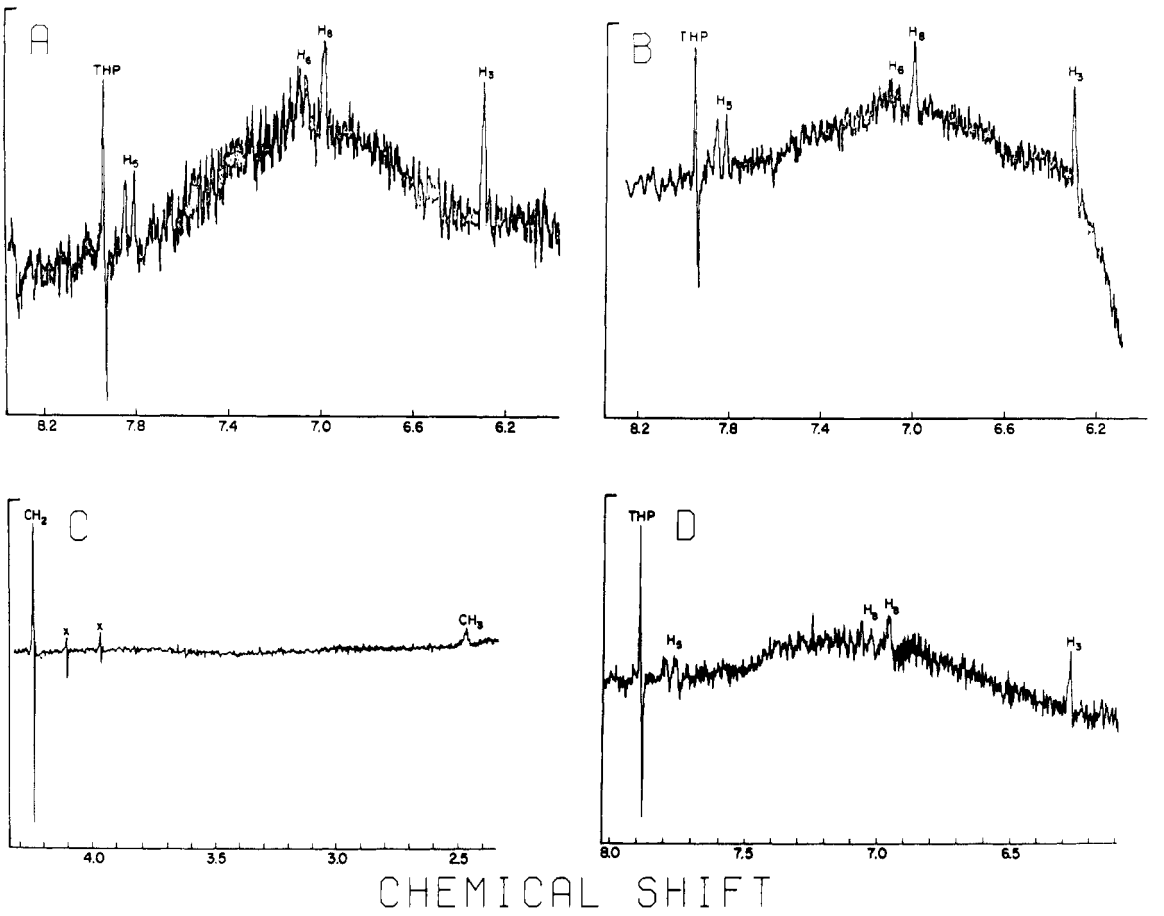

FIGURE 6: (A, B) NOE difference spectra of the aromatic protons of CMU. (A) IgG antibody (1.2×10^{-5} M) + 1 mM CMU; the irradiating frequency was at 1.01 ppm from TSP. (B) IgG antibody (1.2×10^{-5} M) + 1 mM CMU; the irradiating frequency was at 2.07 ppm. (C) The NOE difference spectrum of the aliphatic protons of CMU: 1.2×10^{-5} M IgG antibody + 1 mM CMU; the irradiating frequency was at 0.70 ppm. (D) Fab (1.1×10^{-4} M) + 1 mM CMU; the irradiating frequency was at 2.04 ppm.

TABLE VI: Nuclear Overhauser Effect on the CMU Protons.

	% NOE	
	In IgG antibody[a]	In Fab[b]
H_3	−31	−25
H_8	−23	−24
H_5	−18	−19
H_6	−12	−13
CH_3	−10	
CH_2	0	

[a] *On resonance* was set at 1.95 ppm and *off resonance* was set at −2.11 ppm from TSP. From 1 mM CMU + 1.2×10^{-5} M IgG antibody in 0.15 M PBS in D_2O at pD 7.6. [b] *On resonance* was set at 2.04 ppm and *off resonance* was set at −0.93 ppm. From 1 mM CMU + 1.1×10^{-4} M Fab in 0.15 M PBS in D_2O, pD 7.6.

6A, where an irradiating radiofrequency field centered at 1 ppm from TSP was applied, and in Figure 6B, where the irradiating frequency was set at 2 ppm from TSP. The same results were obtained whichever part of the aliphatic region of the antibody was irradiated. The introduction of a strong radiofrequency field for irradiation generates a Bloch–Siegert shift (Bloch and Siegert, 1940) in the spectrum such that the "on resonance" spectrum does not exactly match the "off resonance" spectrum resulting in a misalignment in the difference spectrum. The shift is slight, however, so that the difference of broad lines, such as the CMU proton signals in the presence of the antibody, does not show much of an effect. The terephthalic acid internal aromatic reference line, being much sharper, is more sensitive to misalignment, but the integrated intensity of the positive and negative peaks is zero.

A sizable negative NOE for the methyl proton was observed, but the effect on the methylene proton was negligible. This is shown in the difference spectrum in Figure 6C where the irradiating frequency was set at 0.7 ppm from TSP. The negative NOE values as measured from the decrease in peak heights are summarized in Table VI. There was also an intramolecular negative NOE for the aromatic proton signal from the antibody when any part of the aliphatic region was irradiated.

Similar results were obtained with the Fab fragment, but the effect was more selective with respect to irradiation. Irradiation in the aliphatic region at 1 ppm from TSP produced only the intramolecular negative NOE for the Fab, and no significant effect on the hapten protons was observed. However, irradiation at 2 ppm or greater gave a sizable negative NOE on the hapten signals (Figure 6D). The values are listed in Table VI. It was not possible to observe the effect for the methyl or the methylene protons, since the observing and irradiating frequencies were too close.

Discussion

The CMU-anti-CMU antibody interaction should be suitable for study by fluorescence energy transfer, since the emission spectrum of the antibody at 340 nm overlaps the excitation spectrum of the hapten centered at 327 nm. However, the hapten fluorescence is quenched in the bound state, so that any energy transferred would be reemitted inefficiently by the hapten, even if the overlap resulted in an effective energy

transfer. Moreover, there seems to be little energy available for transfer, since the titration of the antibody with the hapten gave only 10% quenching of the tryptophan fluorescence in contrast to the 72% tryptophan quenching reported for the DNP system (Velick et al., 1960). The implications are that no tryotophan residues are in the vicinity of the combining site or that the oscillators are not properly oriented for optimal energy transfer; the NMR results discussed below favor the former interpretation. This finding and the observation that quenching of the hapten occurred in dioxane–water mixtures suggests a nonpolar environment in the active site. Fluorescein shows similar quenching behavior when bound to its specific antibody (Lopatin and Voss, 1971), whereas other fluorophores yield fluorescence enhancement (Parker et al., 1967a,b; Yoo and Parker, 1968; Yoo et al., 1970). The thermodynamic calculations also support the nonpolar nature of the antibody combining site, since the hapten–antibody reaction is entropy driven (Kauzman, 1959).

The differential line broadening and the negative NOE studies of the CMU protons in the presence of either the IgG antibody or the Fab fragment can be interpreted on the basis of the proposed mode of binding shown in Figure 7. The lack of any large chemical shifts suggests that there are no *aromatic rings* in the combining site which could interact with the hapten protons. The presence of aromatic residues in the periphery of the active site cannot be ruled out, since the α or β protons of these residues could interact with the hapten protons, in which case the ring current shift would not be easily detectable. The *methylene protons* are not broadened and give no significant NOE. These results suggest that the group, which is part of the side chain by which the hapten is coupled to the bovine γ-globulin carrier, is in free motion and outside of the binding site.

The differential negative NOE of the CMU *ring protons* decreased in the order $H_3 > H_8 > H_5 > H_6$ which suggests that the hapten fits into the binding site such that the lactone ring is in intimate contact with the base of the combining site and the H_6 proton protrudes slightly above the cavity of the combining site. Hydrogen bonding to the CMU carbonyl oxygen could possibly strengthen the interaction in addition to the hydrophobic forces contributed by the other substituents. On the basis of the chemical structure of the hapten, the H_5 proton should give the least negative NOE due to the possibility of a greater intramolecular contribution to dipolar relaxation from the H_6 and methyl protons which are flanking the H_5 proton. However, the line broadening results show that the H_5 proton has the greatest dipolar relaxation, and it is the H_6 proton that gives the least negative NOE. This finding may be explained by the protrusion of the H_6 proton from the combining site, which removes it from an effective intermolecular dipolar interaction.

The line broadening of the CMU ring protons decreased in the order $H_5 > H_6 > H_8 > H_3$. These findings are consistent with the negative NOE results suggesting that the intramolecular contributions to dipolar relaxation are greatest for the H_5 and H_6 protons which can cross-relax each other. The additional relaxation from the methyl protons would give the H_5 proton the most broadened resonance. On the other hand, the H_8 and H_3 protons are far removed from the other ring protons, so that the dipolar relaxation most likely comes from the intermolecular interactions with the protons in the antibody binding site. This is supported by the finding that the H_8 and H_3 protons have the largest negative NOE. Calculations of the changes in the relaxation rates of the H_6 and H_8 protons on going from the free to the bound state show that the H_6 proton is significantly broadened ($\Delta\nu \sim 1.2$ Hz) by the intramolecular

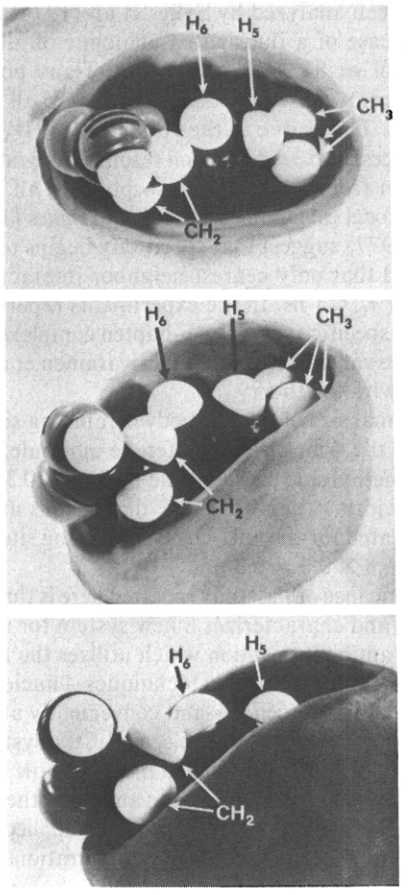

FIGURE 7: Model of the binding of CMU to its specific antibody.

dipolar interaction with the H_5 proton, whereas the H_8 proton is not measurably broadened by the interaction with the H_6 proton ($\Delta\nu \sim 4.5 \times 10^{-2}$ Hz). Since the H_8 proton is broadened by as much as 2 Hz, the relaxation must come from the intermolecular interactions with the neighboring protons in the binding site. A similar type of interaction was reported by Navon and Lanir (1972) who showed that intermolecular dipolar contributions dominated the relaxation rate of sulfonamide inhibitors bound to bovine carbonic anhydrase.

The *methyl protons'* signal shows both broadening and a sizable negative NOE. Despite the internal rotation of the methyl group, the line broadening was on the order of 2 Hz. Intramolecular interactions contributing to this effect would most likely come from the H_5 proton. A -10% NOE for a methyl group is unusually large, so that the intermolecular contribution to dipolar relaxation is important. In a comparable situation, Burgen et al. (1967) suggested that the observed broadening of the methyl signal of tetramethylammonium ions on binding with antibody to phenoxycholine is due to some barrier to rotation of the methyl group.

Saturation of the Fab resonances at 2 ppm from TSP resulted in a negative NOE of the CMU protons, whereas no effect was observed when the irradiation frequency was set at 1 ppm from TSP. This finding indicates the absence of methyl groups in the binding site and suggests the presence of methylene groups in the combining site. Similarly, in the enzyme studies by James (1976), the negative NOE observed on the H_2 proton of adenosine diphosphate on binding with creatine kinase was attributed to the β and α methylene protons of an arginyl residue.

The data reported here also bear upon the question of the specificity of the nuclear Overhauser effect in macromolecules,

which has been analyzed by Sykes et al. (1974). They noted that in the case of a fluorinated analogue of alkaline phosphatase (mol wt 84 000), irradiation of any portion of the proton spectrum caused the disappearance of all fluorine signals equally. This is due to the very efficient W_0 nuclear relaxation process ($\alpha\beta \rightleftarrows \beta\alpha$) which results in the rapid diffusion of excitation from the site of absorption to all parts of the molecule. Model calculations by Hull and Sykes (1975a,b) and by Gerig (1977) suggest that specificity begins to be lost if $\tau_c > 10$ ns and that only nearest neighbor interactions will be important if $\tau_c < 1$ ns. In the experiments reported here, we observed no specificity with IgG–hapten complexes, for which the literature value of τ_c is 32 ns (Kaivarainen et al., 1973), in agreement with the theory.

The minimal size of the antibody combining site needed to accomodate the 4-methylumbelliferone molecule, without the *O*-carboxymethyl side chain, would be 0.83 × 0.35 × 0.68 nm (length × width × depth). These dimensions are similar to those calculated for the anti-DNP combining site by Dwek et al. (1977): 0.8 × 0.3 × 0.3 nm.

The significance of the study reported here is threefold. First, it describes and characterizes a new system for investigating the hapten–antibody reaction which utilizes the full potential of two powerful spectroscopic techniques—nuclear magnetic resonance and fluorescence—and conveniently allows the use of radioisotopic tracers as well. Secondly, the system has been used to construct a rather detailed model of how the hapten is held in the antibody combining site and what the structure of the site may be. Finally, this system can be applied to the study of membrane receptors in vesicle preparations or in solution.

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