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Relation between Structure and Specificity of Antibodies: Nuclear Magnetic Resonance Study of Binding Fluorine-19 Labeled Nitrophenyl Haptens to Myeloma Immunoglobulins M315, M460, and X25†

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ABSTRACT: The relation between structure and specificity of antibodies has been explored by ^{19}F NMR studies of the binding of trifluoromethyl analogues of nitrophenyl haptens to the three mouse myeloma immunoglobulins M315, M460, and X25. We have used haptens with trifluoromethyl groups located at the ortho or para positions of the phenyl ring or attached to the side chain, two atoms removed from the ring (i.e., $-NHCH_2CF_3$). The changes in chemical shift between hapten free in solution and bound to antibody are sensitive to microenvironment and range from 1.7-ppm downfield to 1-ppm upfield. The shifts of *p*-trifluoromethylnitrophenyl haptens bound to M315 and M460 are both large downfield shifts, which are likely caused by van der Waals interaction and ring-current effects, particularly from tyrosine-34 (L); these haptens do not show similar shifts when bound to X25 which has a deletion of tyrosine-34 (L). Other differences in the binding of the aromatic rings of haptens by M315, M460, and X25 are observed and their origins considered. The importance of hydrogen bonding in the thermodynamic affinity of antibody for hapten has been estimated by comparisons of binding affinities for haptens with trifluoromethyl groups in place of nitro groups.

In their recognition of foreign substances, antibodies display both a high degree of specificity and a wide diversity (Richards and Konigsberg, 1973) whose molecular origins have been probed by a variety of physical techniques (Nissonoff et al., 1975). Complementarity between hapten and binding site plays an important role in the antibody–antigen interaction (Kabat, 1976), though we do not as yet have a sophisticated understanding of the "strange cross reaction" of apparently dissimilar haptens for the same antibody (Michaelides and Eisen, 1974). A molecular understanding of the interaction between antibody and antigen (or hapten) should help to resolve some of these questions and might also serve as a preliminary basis for subsequent elucidation of the way in which binding of antigen to certain classes of antibodies triggers the various effector functions (Spiegelberg, 1974) such as the complement cascade (Müller-Eberhard, 1968) and lymphocyte transformation (Vitetta and Uhr, 1975).

Homogeneous proteins (Potter, 1972) secreted by myeloma tumors provide an ideal subject for molecular studies of antibody–antigen interactions. Many of these proteins have been screened by precipitin assay against a variety of antigens (Potter, 1971), and groups of proteins have been identified which exhibit specificities for substances related to phosphorylcholine (Leon and Young, 1970), dinitrophenol (Schubert et al., 1968), and various oligosaccharides (Vicari et al., 1970).

The three plasmacytomas studied grow in BALB/c mice and produce IgA type antibodies secreted primarily as oligomers ($[H_2L_2]_n$; n = 2–5 or more). M315 has a λ_2 light chain (Dugan et al., 1973), whereas M460 and X25 have a κ light chain (Jaffe et al., 1971; Sharon and Givol, 1976). M315 has a high affinity for 2,4-dinitrophenyl (DNP)[1] and 2,4,6-trinitrophenyl (TNP) haptens ($K_{assoc} \approx 10^6$ to 10^7 M^{-1}) (Eisen et al., 1968) and also binds menadione (vitamin K_3) with an affinity of 10^5 M^{-1}, a "cross reaction" of the type mentioned earlier. The binding specificity of M315 with a variety of other haptens has also been studied (Haimovich and Eisen, 1971). M460 binds nitrophenyl haptens with moderate affinity ($K_{assoc} \approx 10^5$ M^{-1}), exhibits high affinity for 2,4-dinitronaphthol ($K_{assoc} \approx 10^7$ M^{-1}) (Haimovich et al., 1972), and binds menadione with an affinity comparable to that for nitrophenyl haptens (Johnson et al., 1974). X25 displays a moderate af-

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[1] Abbreviations used are: BGG, bovine γ-globulin; DNP, 2,4-dinitrophenyl; EDTA, ethylenediaminetetraacetate; NMR, nuclear magnetic resonance; ppm, parts per million; $NaDodSO_4$, sodium dodecyl sulfate; TNP, 2,4,6-trinitrophenyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; GABA, γ-aminobutyric acid.

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finity for dinitrophenyl haptens ($K_{assoc} \approx 10^5$ to 10^6 M^{-1}) but does not bind menadione appreciably ($K_{assoc} < 10^3$ M^{-1}) (Vine, W., et al., 1978, unpublished communication).

The complete variable-region amino acid sequence of M315 has been determined (Francis et al., 1974; Dugan et al., 1973) as has the sequence of M460 through the first hypervariable region (Hunkapiller, M. W., Hardy, R. R., Hood, L., and Richards, J. H., unpublished observations). For X25, a fragmentation scheme yielding protein comprising the variable domains of the heavy and light chains has been presented (Sharon and Givol, 1976), and the sequence through the first hypervariable region has been determined (Hunkapiller, M. W., Hardy, R. R., Hood, L., and Richards, J. H., unpublished observations).

Nuclear magnetic resonance serves as an extremely sensitive technique for the the study of microenvironments in proteins (Roberts and Jardetsky, 1970). Both ^{31}P and ^{13}C NMR techniques have been used to investigate myeloma proteins which bind phosphorylcholine and its analogues (Goetze and Richards, 1977a,b, 1978; Gettins et al., 1977). Binding to M315 has been studied by a number of NMR techniques, including observations of ^{1}H (Dwek et al., 1975, 1977) and ^{19}F (Kooistra and Richards, 1978). This latter work used fluorinated nitrophenyl haptens in which one of the nitro groups attached to the phenyl ring was replaced by a trifluoromethyl group. Such substitution might not greatly change the interaction between hapten and antibody, for the electronegativities of both groups are similar (Wells, 1968) (3.35 for trifluoromethyl compared to 3.4 for nitro) and their sizes are comparable (Coles and Hughes, 1949). In addition, the red shift observed when DNP hapten binds to nitrophenyl-specific antibody (Eisen et al., 1968) is also seen when the trifluoromethyl analogues are bound by these antibodies (Hardy, R. R., unpublished observation). A major difference between a nitro and a trifluoromethyl group is their relative tendency to act as acceptors in the formation of hydrogen bonds (Doddrell et al., 1969) ($NO_2 \gg CF_3$), a difference which can be used to probe the importance of hydrogen binding in the antibody-hapten interaction.

The present work extends the previous ^{19}F NMR study of the binding to M315 of fluorinated nitrophenyl haptens to two other antibodies with nitrophenyl specificity, M460 and X25. In addition, we have examined the binding of a hapten in which a fluorine label has been located on the aliphatic side chain.

Materials and Methods

Tumor Maintenance. MOPC-315 was obtained in solid form from the Salk Institute for Biological Studies, Cell Distribution Center (San Diego, Calif.). MOPC-460 and XRPC-25 were generously provided by Dr. Michael Potter, National Institutes of Health (Bethesda, Md.). The tumors were transplanted by subcutaneous injection into female BALB/c mice of approximately 1-mm^3 pieces of minced tumor tissue (Potter et al., 1972). Mice were obtained from the Texas Inbred Mouse Co. (TIM Co., Houston, Texas).

Purification of Myeloma Protein. Protein was isolated from the ascites of mineral oil primed CDF1 hybrid females (BALB/c × DBA/2) which had been injected with a single cell suspension of the tumor (Potter et al., 1972). This suspension was obtained by pressing the solid tumor (1 cm^3) through a fine (200 mesh) screen. Within 2 or 3 weeks, ascites developed and could be removed through a needle puncture twice weekly. Mice usually survived 2 or 3 weeks after the initial tapping. Myeloma protein yields of 2–3 mg/mL were obtained from the pooled ascites fluid. After collection, cells were centrifuged, and the supernatant was poured off and stored frozen at −20 °C.

Prior to reduction and alkylation, the myeloma protein could be assayed by immunoprecipitation with DNP-BGG either by ring test or agar diffusion (Ouchterlony).

Myeloma protein was purified by affinity chromatography according to a modification of a published procedure (Inbar et al., 1971). Pooled ascites was made 0.2 M in Tris by adding 0.1 volume of 2 M Tris (pH 8.6). The solution was made 0.01 M in dithiothreitol and stirred for 1 h at room temperature under an argon atmosphere. The pH was then adjusted to 7.8 by adding 0.1 volume of 2 M Tris (pH 7.2), and the solution was chilled to 0 °C in an ice bath. The solution was made 0.03 M in iodoacetamide and stirred for 30 min under argon at 0 °C. The reduced and alkylated protein was then applied to a DNP affinity column.

The affinity column was prepared by coupling 1,6-diaminohexane to cyanogen bromide activated Sepharose 4B. This primary amine spacer arm gel was then reacted with 2,4-dinitrofluorobenzene to yield the DNP-Sepharose conjugate.

Bound myeloma protein was eluted from the affinity column with a 0.05 M DNP-Gly solution (Goetzl and Metzger, 1970). Eluate from the column passed directly to a small Dowex 1-X8 ion-exchange resin column (2.5 × 4 cm) which removed DNP-Gly from the protein. The amount of protein was monitored by its absorbance at 280 nm; the concentration was determined by using an absorbance value of 1.35 for a 0.1% protein solution (1-cm path length) (Underdown et al., 1971).

$NaDodSO_4$ gel electrophoresis showed some contamination by albumin, and the myeloma protein was further purified on Whatman DE-52 ion-exchange cellulose. The solution of 7S monomer was dialyzed against 0.03 M potassium phosphate (pH 8.0) buffer and applied to the DE-52 column equilibrated with this buffer. The IgA fraction was eluted with a 0.03 to 0.3 M potassium phosphate linear gradient (pH 8.0) at 4 °C. The IgA eluted first, followed by the albumin.

Preparation of Fab' Fragments. Fab' fragments were made by a 1% pepsin digest of purified 7S monomer (Inbar et al., 1971). Purity was assayed by 7.5% $NaDodSO_4$ gel electrophoresis which showed that greater than 90% was Fab'.

Preparation of NMR Samples. Purified Fab' protein was dialyzed against buffer (0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA, 0.02% NaN_3, pH 7.40) and brought to 30 mg/mL (0.54 mM) by pressure dialysis (AMICON Diaflo). Concentration was determined by measuring absorbance at 278 nm (1.4 for a 0.1% protein solution, 1-cm path length) (Inbar et al., 1971). The fluorinated haptens were added in aliquots of 0.05 M stock solution. Haptens were dissolved in the buffer described above. The $-NHCH_2CF_3$ hapten was insoluble in aqueous solution and was therefore prepared as a stock solution in acetone.

Fluorine NMR spectra were obtained with 2-mL protein samples in 12-mm flat-bottom NMR tubes containing a 5-mm coaxial tube of D_2O for locking. Spectra were observed on a Varian XL-100-15 at 94.1 MHz.

Preparation of Haptens. The following haptens were all synthesized by a modification of the published procedure described by Porter (1950): γ-*N*-(2,6-dinitro-4-trifluoromethylphenyl)aminobutyric acid [the *p*-CF_3TNP analogue], γ-*N*-(2-nitro-4-trifluoromethylphenyl)aminobutyric acid [the *p*-CF_3DNP analogue], and γ-*N*-(4-nitro-2-trifluoromethylphenyl)aminobutyric acid [the *o*-CF_3DNP analogue]. Details of synthesis and physical properties were reported previously (Kooistra and Richards, 1978).

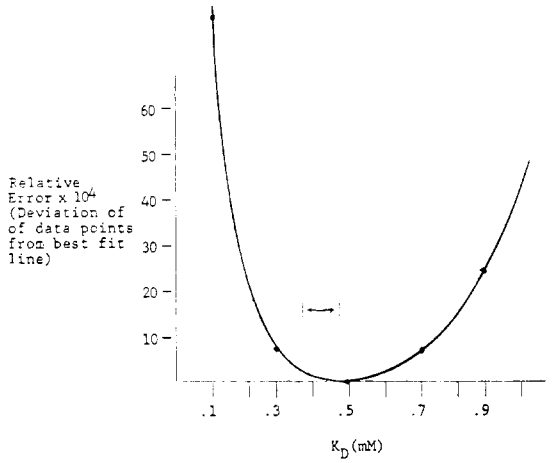


FIGURE 1: Variation in error as a function of dissociation constant assumed. Error bar represents ±5% variation in Δ, ±15% variation in K_D.

2-N-(2,4-Dinitrophenyl)aminotrifluoroethylene. This hapten was prepared by the reaction of 2,2,2-trifluoroethylamine with 2,4-dinitrofluorobenzene in an ethanol–10% bicarbonate solution (2:1, pH 9) for 12 h at room temperature. The ethanol was removed in vacuo and the solution was extracted with ether. The ether solution was washed with 10% HCl and then water, dried over $MgSO_4$, and the ether removed in vacuo. The resulting solid was recrystallized from ethanol to give yellow needles: mp 116 °C; IR 3360 (N–H stretch), 1630 and 1540 (C=C), 1340 (C–N stretch), 1290 (CF_3), 1160, 1120, and 830 cm^{-1} (aromatic unsymmetrical substitution); ^1H NMR (acetone-d_6) δ 9.0 (d, 1 H), 8.4 (d of d, 1 H), 7.55 (d, 1 H), 4.57 (m, 2 H, br NH under ar peak); ^{19}F NMR (acetone-d_6) 3.19 ppm (downfield from F_3AcOH, t, J = 8.7 Hz); absorption maximum 343 nm; molar absorptivity 2×10^4 cm^{-1} M^{-1}. Anal. Calcd for $C_8H_6N_3O_4F_3$: C, 36.23; H, 2.26; N, 15.88. Found: C, 36.28; H, 2.27; N, 15.67.

Treatment of Data. For NMR peaks in fast-exchange on the NMR time scale, the observed chemical shift is a weighted average of the shift in the two environments (free and bound). Thus:

$$\delta = \frac{[\text{AbHap}]}{[\text{Hap}_0]}\Delta$$

where δ is the chemical-shift difference between the free and bound peak, [AbHap] is the concentration of bound hapten, and $[Hap_0]$ is the total hapten concentration. By observing the chemical shift at a number of ratios of hapten to antibody one can evaluate both Δ and K (Gammon et al., 1972). This method was used in all cases to determine the bound chemical shifts and the association constants for the fluorinated haptens. An assumed K is used to calculate [AbHap] for a given $[Hap_0]$ and $[Ab_0]$. A plot of chemical shift vs. $[AbHap]/[Hap_0]$ will be linear for the best K with slope of Δ. Figure 1 shows the deviation from linearity when K is varied. A corresponding variation in Δ is also indicated. Relative error is defined as the best r^2 value from a linear least-squares fit minus the r^2 for a given K_D divided by the best r^2 value obtained for that data set. The best r^2 value for a set of six or more data points was typically 0.99 or better.

The method is accurate so long as the binding constant is of the same order of magnitude as the protein concentration employed. In all but two cases (the $-NHCH_2CF_3$ hapten binding to M315 and X25), this criterion was met. The lower accuracy for the other two values is indicated in Table I by the use of only one significant figure.

TABLE I: Binding Affinities of DNP-Specific Myeloma Proteins (at 25 °C).

	K_{assoc} (M^{-1})		
	X25	M460	M315
DNP-Lys	1.9×10^5 [b]	6.7×10^4 [b]	2.4×10^6 [a]
menadione	$<5 \times 10^3$ [b]	1×10^4 [b]	1.6×10^5 [b]
2,4-dinitronaphthol	$<3 \times 10^4$ [b]	3.4×10^5 [b]	6.7×10^4 [b]
p-CF_3DNP analogue	2.0×10^3	4.4×10^3	7.8×10^5 [a]
p-CF_3TNP analogue	6.7×10^3	2.3×10^3	3.4×10^6 [a]
o-CF_3DNP analogue	6.4×10^3	1.0×10^3	1.3×10^5 [a]
chain CF_3DNP	2×10^5 [c]	5×10^3	2×10^5 [c]

[a] Fluorescence quenching D. A. Kooistra. [b] R. Hardy and W. Vine, equilibrium dialysis; remaining determined by ^{19}F NMR data. [c] Due to the high affinity constant less certainty in these values determined by NMR ($K \geqslant$ value shown).

Results

Table I lists the affinities of M315, M460, and X25 for the various fluorinated haptens determined in this work by the NMR procedure just described and in earlier work by fluorescence quenching (Kooistra and Richards, 1978). For comparison, affinities for other common haptens are given.

Table II compares the relative affinities of the three antibodies for DNP-Lys and the fluorinated haptens in an attempt to assess the relative importance of hydrogen bonding in M315, M460, and X25.

Table III collects the changes observed in the ^{19}F chemical shifts of the haptens between solution and the binding sites of the Fab′ fragments of the antibodies. These values of Δ were determined as previously described. Figure 1 shows how values of K and Δ are related to the observed experimental parameters. The values of Δ calculated in this way range from 1-ppm downfield to 1-ppm upfield for bound hapten relative to hapten in solution. The p-trifluoromethyl analogues of both DNP and TNP have the same Δ values when binding to M315; in contrast, these two haptens have quite different values of Δ when they bind to M460 and when they bind to X25. The p-trifluoromethyl-TNP analogue is in slow exchange between solution and M315, whereas the p-trifluoromethyl-DNP analogue is in intermediate exchange. Other haptens with M315 and all haptens with M460 and X25 are in fast exchange.

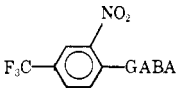
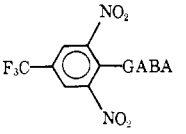
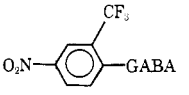
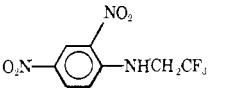
Discussion

Binding Affinities. Formation of hydrogen bonds between the antibody (as donor) and the nitro group of the hapten (as acceptor) likely provides to the hapten–antibody complex a significant stabilization in addition to other stabilizing factors such as charge transfer and hydrophobic and ionic interactions. Therefore, replacement of a nitro group, which forms moderately strong hydrogen bonds (Baitinger et al., 1964), by a trifluoromethyl group, which forms much weaker hydrogen bonds (Doddrell et al., 1969), should reduce the stabilization of the antibody complex. The degree to which this occurs may provide some measure of the importance of such hydrogen bonding in the affinity of antibody for hapten. Unless the binding site is very narrow, the greater bulk of the spherical trifluoromethyl group compared to the planar nitro group should not, itself, appreciably affect binding (Coles and Hughes, 1949). Also, as mentioned previously, the red shift observed on binding the fluorinated haptens thought to be due to a charge-transfer interaction betwen hapten and tryptophan in the antibody is the same as for corresponding nitrophenyl haptens (Hardy, R. R., 1978, unpublished observation).

TABLE II: Comparison of Unitary Free Energies of Binding Fluorinated Haptens with Binding of DNP-Lys (at 25 °C).

	X25		M460		M315	
hapten	$-\Delta G_u$ (kcal/mol)	$\Delta G_u/\Delta G_u^{DNP\text{-}Lys}$	$-\Delta G_u$ (kcal/mol)	$\Delta G_u/\Delta G_u^{DNP\text{-}Lys}$	$-\Delta G_u$ (kcal/mol)	$\Delta G_u/\Delta G_u^{DNP\text{-}Lys}$
DNP-Lys	9.59	1.0	8.97	1.0	11.1	1.0
p-CF_3DNP analogue	6.89	0.72	7.35	0.82	10.4	0.94
o-CF_3DNP analogue	7.58	0.79	6.48	0.72	9.36	0.84
chain CF_3DNP	9.61	1.0	7.43	0.83	9.61	0.86

TABLE III: Δ Chemical Shifts (in ppm) Bound–Free.[a]

protein				
M315	1.8[b]	1.8[b]	0.6[b]	−0.77
M460	0.56	1.0	0.33	0.94
X25	0.36	0.05	−0.99	−0.54

[a] A minus sign indicates upfield relative to free hapten. [b] From Kooistra and Richards (1978).

The concept of unitary free energy of binding provides a useful basis for comparing binding interactions. As comparisons of unitary free energies of binding effectively eliminate the term for the entropy of mixing, they are more appropriate for discussion of the energetics of binding than simple free-energy values. Equation 1 states the relationship (Utsumi and Karush, 1964) which has previously been used to compare the binding of inhibitors to papain (Berger et al., 1970) and in the study of antibodies against poly(phenylalanine) (Schlecther, 1971). Table II summarizes the unitary free-energy values for binding DNP-Lys and the three trifluoromethyl haptens; the percent binding relative to DNP-Lys is also shown. These values for M315 indicate that hydrogen bonding with a group in the ortho position of the phenyl ring or attached to the side chain is stronger than that with a group in the para position. Hydrogen-bonding interactions from the para position and from chain substituents with M460 are similar to those from the ortho position with M315. However, interactions of M460 are stronger with substituents in the ortho position than with substituents in the para position or on the chain. X25 shows important differences in the way it hydrogen bonds with haptens; the para interaction is strongest, and no interaction with substituents on the chain is apparent. Table IV summarizes the relative hydrogen bonding interactions for M315, M460, and X25.

$$\Delta G_u = -1.365 \log K_A - 2.38 \quad (1)$$

Chemical Shifts—Hapten Environments. In the absence of an X-ray structure, the explanations of chemical-shift data must necessarily be tentative and somewhat speculative. Two major effects are likely to influence fluorine shifts on binding: van der Waals interaction and ring currents. Van der Waals effects arise from crowding of the trifluoromethyl group which distorts the π electron cloud and causes downfield shifts (Farnum and Patton, 1973). Ring currents, which arise from local magnetic fields about π ring systems, can cause either upfield or downfield shifts, depending on the orientation of the aromatic ring relative to the probe nucleus (Emsley et al., 1965).

Assignment of fluorine chemical shifts may be approached by an examination of the known facts regarding DNP binding myeloma proteins:

TABLE IV: Contribution of Hydrogen Bonding to Binding.[a]

	M315	M460	X25
p-CF_3	w	m	s
o-CF_3	m	s	m
chain CF_3	m	m	a

[a] Abbreviations used: w, weak; m, moderate; s, strong; a, absent.

(1) Affinity labeling of M315 has localized tryosine-34 (L) at the DNP ring binding subsite and lysine-52 (H) at the end of the aliphatic chain (Goetzl and Metzger, 1970; Haimovich et al., 1972).

(2) A positive subsite consisting of arginine-95 (L) and/or lysine-52 (H) interacts with the carboxylate end of the hapten (Haselkorn et al., 1974).

(3) Circular dichroism and optical absorbance studies indicate that a charge-transfer complex is formed between the DNP ring and a tryptophan residue (Freed et al., 1976; Eisen et al., 1968).

(4) For a number of immunoglobulins, whose X-ray structures are known, a large ordered tertiary structure (the "immunoglobulin fold") seems always to be present (Padlan and Davies, 1975).

Using such structural data and the known amino acid sequence, Padlan has constructed a model of the M315-binding site (Padlan et al., 1976) which has been refined as the results of NMR studies (Dwek et al., 1977). Since M315, M460, and X25 show considerable sequence homology for the regions sequenced, this structural model might be extended to these other DNP-binding proteins and amino acid substitutions (or deletions/insertions) correlated with the environments experienced by bound haptens.

Comparison of the first hypervariable light-chain region (Figure 3) of M315 and X25 shows only one major difference: the deletion of tyrosine-34 (L). Both M315 and X25 have a high affinity for DNP and both produce similar red shifts in the hapten absorption spectrum on binding (indicating a charge-transfer interaction). If one assumes that the observed sequence homology between M315 and X25 continues past the

		para CF_3 shift (ppm)	
		DNP	TNP
M315	Thr-Ser-Asn-Tyr-Ala-Asn-Trp	1.8	1.8
X 25	Ile-Ser-Asn-Asn-Leu-His-Trp	0.36	0.05
M315	Thr-Ser-[ ]-Asn-[ ]-Tyr-Ala-Asn-Trp	1.8	1.8
M460	Ser-Asn-Gly-Asn-Thr-Tyr-Leu-His-Trp	0.6	1.0

FIGURE 2: L1 hypervariable sequences and ^{19}F shifts.

		ortho CF_3 shift	chain CF_3 shift
X 25	Thr-Ser-Gly-Tyr-[ ]-Trp-Asn-Trp-Ile	-1.0	0.5
M315	Thr-Ser-Gly-Tyr-Phe-Trp-Asn-Trp-Ile	-2.5	-0.8
M460	Thr-Ser-Gly-Tyr-[ ]-Met-Asn-Trp-Ile	-0.5	-1.2

FIGURE 3: H1 hypervariable sequences and ^{19}F shifts (− indicates upfield shift).

40 residues already sequenced, a similar binding cavity should be formed in the two proteins. Thus, the binding-site residues near the *p*-trifluoromethyl group are in the L1 region at the "bottom" of the pocket. Binding sites in both M315 and X25 appear to be rigid, so apparently the deletion of tyrosine-34 (L) accounts for the greatly reduced shift of the *p*-trifluoromethyl group in the hapten bound to X25. Such a shift in M315 could arise either from ring currents or from a combination of ring currents and van der Waals effects.

Further clarification of the origin of the ^{19}F chemical shift observed when hapten binds to antibody can come from simultaneous observations of ^{19}F and ^{1}H shifts (Millet and Raftery, 1972). Preliminary studies of a hapten with a CHF_2 group in the para position (Kooistra, D. A., Hardy, R. R., and Richards, J. H., manuscript in preparation) show that, while the ^{19}F resonance is shifted downfield by 1.7 ppm, the ^{1}H signal is shifted upfield by 1 ppm; this result illustrates the important contribution of van der Waals interactions to ^{19}F shifts.

Examination of the L1 hypervariable region of M460 (Figure 2) tends to support the role of tyrosine-34 (L). The only significant difference between M315 and M460 is the additional length of the M460 loop. Tyrosine-34 (L) is present in both as is the large chemical shift observed when hapten binds. Another interesting fact shown by the shift data for M460 is that DNP and TNP bind to M460 in somewhat different conformations (unlike binding to M315). Such differences could reflect small changes in orientations of the aromatic residues in the binding pocket relative to the trifluoromethyl group or changes in the crowding and resultant distortion experienced by the hapten on binding.

In the binding-site model, H1 (the first heavy-chain hypervariable region) lies toward the rear of the binding site at the edge of the aromatic binding subsite. Figure 3 indicates H1 sequences for M315, M460, and X25 along with the chemical-shift data for CF_3 groups at the ortho position and in the side chain.

As was mentioned previously, van der Waals interactions give rise to a downfield shift, while ring currents cause shifts in either direction, depending on orientation. The ring current induced by an external magnetic field generates a field which opposes the applied field above and below the plane of the aromatic ring but reinforces it elsewhere. Thus, nuclei near the sixfold axis normal to the plane of the ring are shifted upfield while those near the plane of the aromatic ring are shifted downfield (Emsley et al., 1965). Therefore, a large downfield shift may result from steric crowding, or from ring currents, or from a combination of the two. However, an upfield shift should be due to a domination of the shift by ring currents. Thus, for X25 both the ortho and $-NHCH_2CF_3$ shifts are likely due predominantly to ring currents [possibly tyrosine-33 (H) or tryptophan-35 (H)]. The $-NHCH_2CF_3$ fluorine shifts caused by binding both to M315 and X25 seem to be dominated by ring-current effects and, given the homology in H1 between M315 and X25, may be due to the same residue [tyrosine-33 (H) or tyrptophan-35 (H)]. The chain shift for M460 is unusual in that it is large and in the opposite direction from M315 and X25. The M460 site is not as rigid as the M315 site (Hsia and Little, 1973; Wong et al., 1974), so such a large van der Waals shift would not be expected. Possibly, the shift of the CF_3 group in the side chain of the hapten on binding to M460 is due to ring-current effects. Substitution of methionine for tryptophan (as in the M460 H1 region) might be expected to change nearby tertiary structure so that significant differences may characterize orientation of aromatic residues in the binding pocket of M460 compared to M315 and X25.

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Estimation of Transmembrane pH Gradients from Phase Equilibria of Spin-Labeled Amines†

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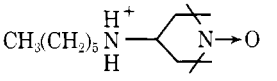
ABSTRACT: Spin-labeled secondary amines have been used to measure transmembrane proton gradients in sonicated liposomes. The electron paramagnetic resonance spectra of these probes show changes in the ratio of membrane associated to free aqueous probe as a function of transmembrane pH gradient. As the pH gradient is increased, inside acidic, the amount of membrane associated probe increases. The results are accounted for by a simple thermodynamic theory.

Gradients of pH across biological membranes are associated with many energy transducing and transport phenomena. As a result, methods which provide a simple and direct means of measuring gradients of H^+ activity have been actively sought.

Estimations of transmembrane pH gradients (ΔpH) are currently obtained by measuring the equilibrium distribution of radioactive weak acids or bases (Addanki et al., 1968; Rottenberg et al., 1972) or by the use of fluorescent amines such as 9-aminoacridine (Deamer et al., 1972). In the latter case, a pH-dependent uptake of the dye is accompanied by a quenching of fluorescence, and a quantitation of ΔpH is achieved by empirical calibration methods.

In this paper, we report on a new approach which has been taken to obtain rapid estimates of ΔpH in sonicated liposomes. By utilizing electron paramagnetic resonance (EPR)[1] spectroscopy, the membrane aqueous solution partition coefficient of spin label I is obtained. It will be shown that this equilibrium



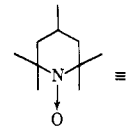
I

is sensitive to transmembrane pH gradients in a manner predicted by a simple thermodynamic theory.

Experimental Section

Materials. Phosphatidylcholine (PC) was prepared from fresh hen eggs according to the procedure of Singleton et al. (1965). The purified phospholipid in chloroform was stored at −20 °C under an argon atmosphere at a concentration of 75 mg/mL. *N,N,N*-Trimethyl-*N*-tempoylammonium bromide was a gift of Carole Hamilton.

Synthesis of N-Hexyl-N-tempoylamine[2] (*I*). 4-Amino-2,2,6,6-tetramethylpiperidinyl-1-oxy (0.50 g; 2.92 mmol) and



≡ tempoyl radical

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[1] Abbreviations used: EPR, electron paramagnetic resonance; PC, phosphatidylcholine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TLC, thin-layer chromatography.

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