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Antibody–hapten interactions in solution

By R. A. Dwek,† R. Jones,† D. Marsh, A. C. McLaughlin,
Elizabeth M. Press,‡ N. C. Price and A. I. White†

†*Oxford Enzyme Group – Science Research Council* and ‡*Medical Research Council Immunochemistry Unit*

Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU

This paper reports the initial progress in a research programme to identify and obtain the relative orientations, in solution, of the amino acid residues that constitute the combining site of the myeloma protein MOPC 315. This protein has a molecular mass of 150000, but enzymic digestion yields the Fv fragment of molecular mass 25000 which still has the combining site intact, as judged by the affinity for dinitrophenyl haptens. Analysis of the e.s.r. spectra of a series of dinitrophenyl spin labelled haptens has allowed the dimensions, rigidity and polarity profile of the combining site to be determined. The combining site is a cleft of overall dimensions 1.1 nm × 0.9 nm × 0.6 nm which has considerable structural rigidity. One of these spin labels has also been used to perturb the n.m.r. spectrum of the Fv and using difference spectroscopy the 270 MHz proton n.m.r. spectrum of the amino acid residues in and around the combining site has been obtained. This spectrum contains only the equivalent of about 30 aromatic and 21 aliphatic protons. Comparison of this difference spectrum with that obtained using a diamagnetic analogue suggests that any conformational changes on hapten binding are mainly localized to the combining site. By the use of (n.m.r.) difference spectroscopy the protons of the three histidine residues in the Fv are observed to titrate with pH and have pK_a values of about 8.1, 6.9 and 6.1. The histidine resonances with pK_a values 6.9 and 6.1 alter slightly in the presence of haptens and also appear in the spin label difference spectrum, and must therefore be in or near to the combining site. These are assigned to His 102_H and His 97_L. The existence of lanthanide binding sites on the Fv, necessary for the mapping studies, has been demonstrated by measurements of Gd III water relaxation rates in Fv solutions and also by the changes in the Fv tryptophan fluorescence on addition of Gd III. At pH 5.5 there is one tight binding site for the lanthanides ($K_D \approx 80$ μM) but in the presence of hapten this is weakened 10–20 fold with a reciprocal effect on the hapten binding. Measurements of the Gd III quenching of the e.s.r. spectrum of a spin labelled hapten bound to Fv indicate that the lanthanide site is *ca.* 1.5 nm from the nitroxide moiety.

1. Introduction

The problem of recognition in solution is a general one throughout biology of which the recognition by antibodies of antigenic determinants is a particularly important example since this is the basis of the body's defence. An antibody directed against an antigenic determinant of a given molecule will react only with this determinant or another very similar structure. This specificity must result from the three dimensional complementarity of the antibody combining site and the antigenic determinant.

Conventionally, problems involving three dimensional structure are attempted using X-ray crystallographic techniques (Poljak *et al.* 1975, this volume). However it is important to realize that crystal formation is essentially a problem of self recognition, that is of a molecule by one

of the same type, while biological recognition is one of recognition of a molecule by one of a different kind. Thus any statements made on the basis of solid studies must be confirmed in solution, for the fear is that because proteins are conformationally flexible the structure in solution could be quite different from the solid. The only technique which can give structural information in solution (in principle comparable to that obtainable from X-ray work) is nuclear magnetic resonance, n.m.r. For structural studies it is obviously desirable to work with homogeneous antibodies, but nearly all conventionally raised antibodies are heterogeneous populations of immunoglobulins. However in certain forms of cancer of immunoglobulin synthesizing cells – known as myelomatosis – homogeneous immunoglobulin molecules – known as myeloma proteins – are produced. These proteins have been shown by their ligand binding properties and many immunochemical parameters to resemble antibodies.

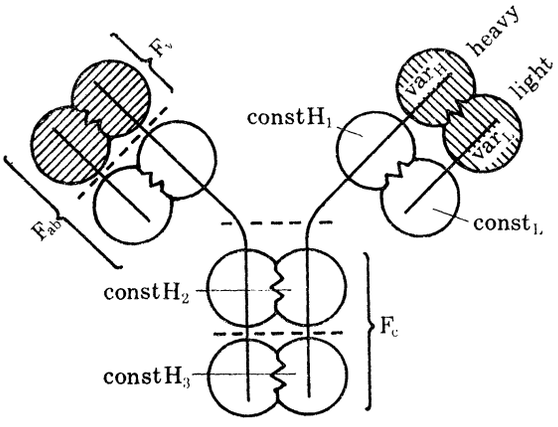

FIGURE 1. Schematic representation of an immunoglobulin molecule showing the various fragments that can be obtained by enzymic cleavage. The molecular mass of the whole unit is 150000 and those of Fab and Fv 50000 and 25000 respectively. The variable regions in the heavy (H) and light (L) chains are shaded.

Amino acid sequence studies on both normal immunoglobulins and myeloma proteins have led to the basic picture of a polypeptide chain structure consisting of two pairs of heavy and light chains joined by disulphide bonds. Each chain has regions of homology 110–120 residues long. The light chain has two such regions and the heavy chain four and the total molecular mass of this basic unit is 150000. The amino acid sequence of the N-terminal region of each of these chains is variable – varying from one antibody molecule to another – whereas the sequence of the rest of the chain is identical within a given class and type.

The variable region contains the antibody combining site. Within this region there are hypervariable regions (Kabat & Wu 1971) and it has been suggested that the basis of antibody specificity involves these regions in the combining site.

Under controlled conditions it is possible to split the antibody molecule into Fab and Fc fragments. The Fab fragment has a molecular mass of 50000 and contains the antigen binding site. The assumption that the binding site was within the variable region of the Fab fragment received formal proof when it was shown that the Fab fragment of a (dinitrophenyl) DNP-lysine binding mouse myeloma protein, MOPC 315, could be further split into an Fv fragment (molecular mass 25000) which was shown to retain full affinity for the ligand (Inbar, Hochman & Givol 1972).

N.m.r. structural determination in proteins is still in a very early stage for the problems of

sensitivity, resolution and assignment are still very major ones. The most ambitious n.m.r. structural determination of a protein so far is that of lysozyme (molecular mass 14400) and although considerable success has already been achieved (Campbell, Dobson, Williams & Xavier 1973) much of the initial work would not have been possible without the X-ray structure. As in X-ray crystallography of proteins the problem of structural determination using n.m.r. methods becomes increasingly more difficult as the size of the molecule increases. However, the smallest antibody fragment that preserves the integrity of the combining site (the Fv fragment of MOPC 315) has a molecular mass of 25000, nearly twice that of lysozyme. In this case attempts to obtain suitable crystals for X-ray studies have as yet been unsuccessful and this provides a further impetus for the n.m.r. studies which can, of course, be carried out in solution.

We have begun a programme to attempt by n.m.r. to identify and assign the amino acids in and near to the combining site of the Fv fragment of MOPC 315 and also to obtain their relative orientations. However to obtain some idea of the magnitude of the problem, our initial aim has been to define the dimensions and rigidity of the combining site. This we have achieved from electron spin resonance (e.s.r.) studies on a series of structurally related DNP-nitroxide spin labelled haptens. Then using one of these haptens we have obtained the 270 MHz n.m.r. spectrum of the resonances of the protons from amino acids near to this hapten. The ultimate assignment of this spectrum should enable the contact amino acid residues to be determined. The actual geometrical orientation of these amino acids requires the presence of at least one other paramagnetic centre (preferably a lanthanide (Dwek 1973)). For in much the same way as X-ray crystallographic structural studies of proteins initially require heavy atoms or reference points so the corresponding n.m.r. studies require paramagnetic centres (Dwek 1973). The final part of the work reported here is thus concerned with the search for intrinsic lanthanide sites.

2. Experimental

Fluorescence measurements were made on a Perkin Elmer–Hitachi MPF 2A spectrofluorimeter (T = 25 °C). All other methods and materials have been described previously (Dwek *et al.* 1975).

3. E.s.r. studies of antibody binding and the combining site

The e.s.r. spectra of dinitrophenyl hapten spin labels have been used to study the myeloma protein MOPC 315 and its hapten combining site. E.s.r. titrations have been used to investigate the hapten binding, and spectra of the bound hapten used to investigate the overall motion of the various antibody fragments: Fv, Fab and whole IgA. A series of structurally related hapten spin labels have been used to probe the dimensions and rigidity of the antibody combining site (from the anisotropy of the spin label motion) and also to probe its polarity profile (using the isotropic hyperfine interactions of the spin label).

(*a*) *E.s.r. binding titrations*

The e.s.r. spectra obtained on titrating the Fv fragment of MOPC 315 with the dinitrophenyl spin label hapten **1** (*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-2,4-dinitrobenzene) are given in figure 2. The spectra are composed of an overlap of the bound and free label. The two can

be distinguished, since the bound label has a spectrum, characteristic of strong immobilization (Smith, Marsh & Schreier-Mucillo 1975) which extends beyond the spectral range of the characteristic three-line spectrum of the free spin label. The spectral overlap is minimal in the regions marked by the arrows in figure 2, and hence these peaks can be used to follow the

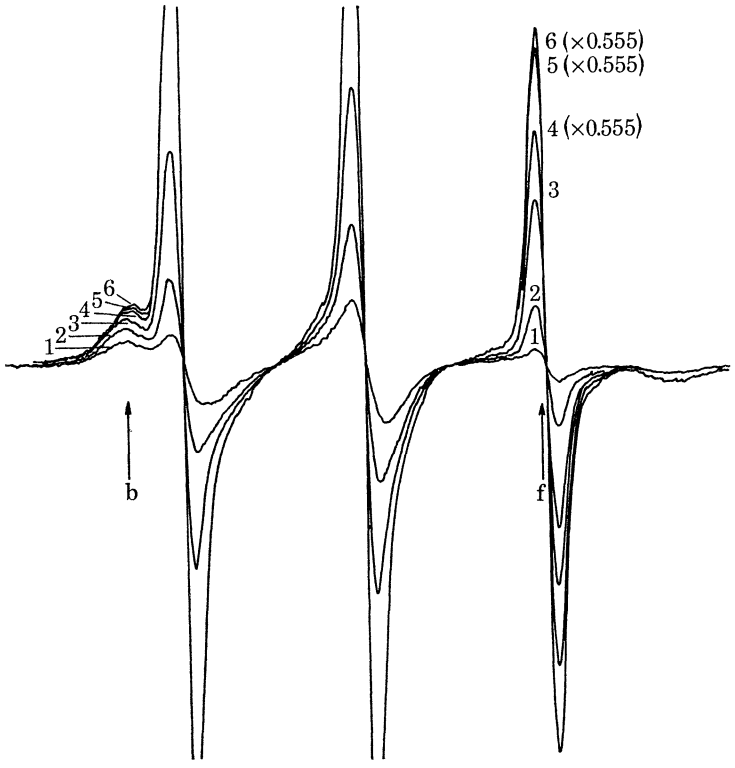

FIGURE 2. E.s.r. spectrum of spin label hapten **1** titrated into the Fv from MOPC 315. The broad lines, b, arise from the strongly immobilized hapten spin label bound to Fv, and the narrow lines, f, arise from the free hapten spin label in aqueous solution. (5 μl aliquots of 1000 μM spin label hapten added to 45 μM Fv in 0.15 M NaCl, pH 7.0; T = 20 °C, modulation = 0.2 mT.)

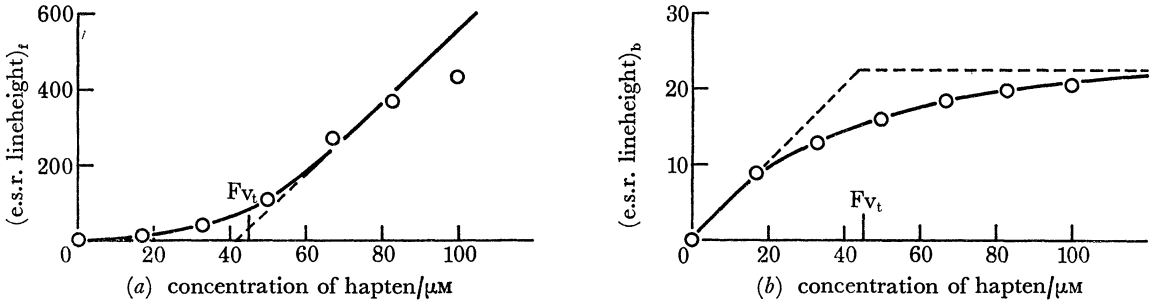

FIGURE 3. Titration curves of (*a*) the free, f, hapten spin label **1** and (*b*) the bound, b, hapten spin label **1** on binding to the Fv of MOPC 315.

titration. The titration curves for the bound and free labels are given in figure 3. Some difficulty is experienced with the limited solubility of the hapten spin label because of the relatively high concentrations required to detect the signal from the bound label. This is seen in the fall-off of the signal from the free hapten at the upper end of figure 3*a*. Combination of the data from figure 3*a* and *b* allows the calculations of dissociation constants for the hapten–Fv complex.

These are in the range 1–4 μM, with the value K_D = 1 μM corresponding to a low concentration of added hapten for which solubility problems are likely to be minimal.

(*b*) *Dimensions and rigidity of the Fv combining site*

The e.s.r. spectra of the different spin label haptens when bound in the combining site of Fv can be examined by reacting the antibody fragment with a slight excess (over the stoichiometric quantities) of the appropriate hapten, and then removing most of the 'free' signal by

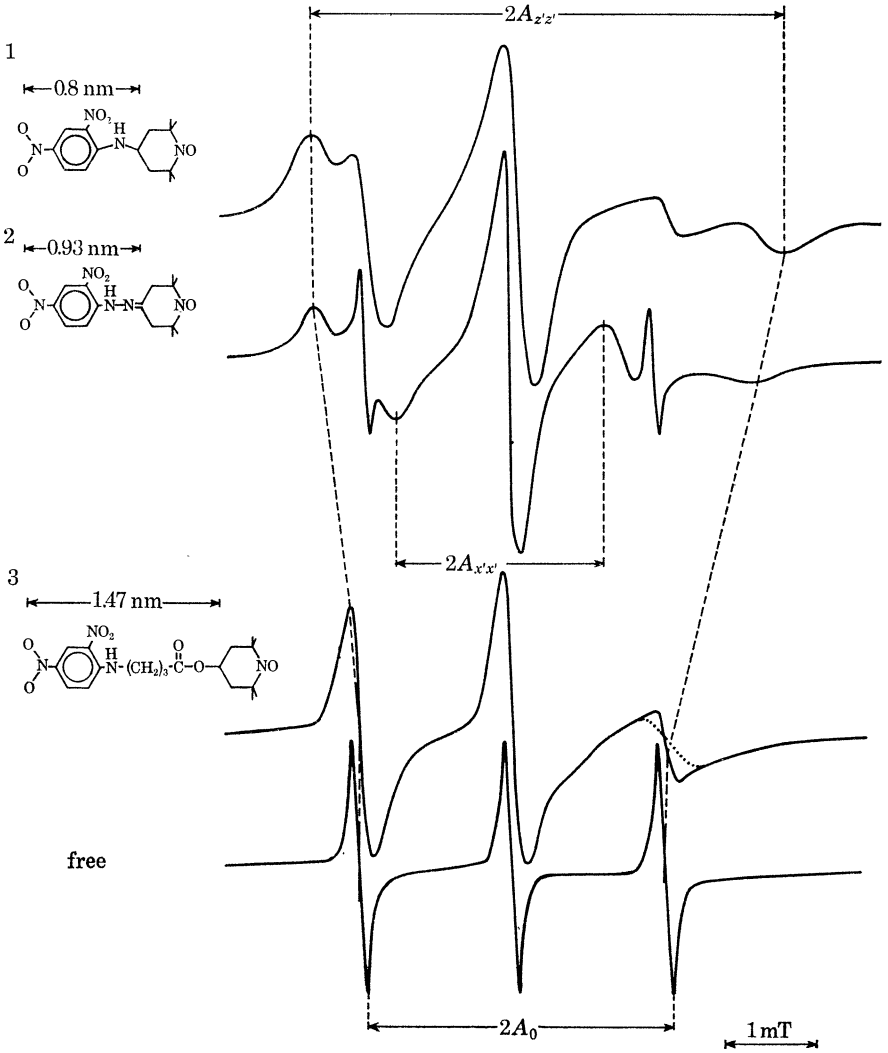

FIGURE 4. E.s.r. spectra of six-membered ring haptens spin labels of various lengths bound to the Fv from MOPC 315. The bottom spectrum is of free spin label in aqueous solution. (10 mg/ml Fv in 0.15 M NaCl, pH 7.0; T = 19 °C, modulation = 0.2 mT.)

dialysis. The spectrum of the spin label haptens in the combining site will be sensitive to both the amplitude and rate of motion of the bound spin label (Schreier-Mucillo *et al.* 1973). Since the amplitude of motion of the bound hapten is limited by the steric effects of neighbouring groups in the antibody combining site and the rate of motion is partially governed by the flexibility of these groups, the spectrum of the bound hapten label is capable of giving information about the dimensions and rigidity of the combining site.

The e.s.r. spectra of the various structurally differentiated dinitrophenyl hapten spin labels which have been used in probing the antibody combining site are given in figures 4 and 5. Comparison of the various spectra in figure 4 shows that as the distance from the nitroxide to the dinitrophenyl ring increases, both the linewidths and anisotropy (or spectral extent) of the spectra decrease. This corresponds to an increased amplitude and rate of motion of the nitroxide group which partially averages out the anisotropy in the spin label e.s.r. spectrum. The limiting case of this is the isolated spin label hapten rotating freely in solution, in which all the anisotropy is averaged out giving rise to a narrow three-line spectrum as seen in figure 4.

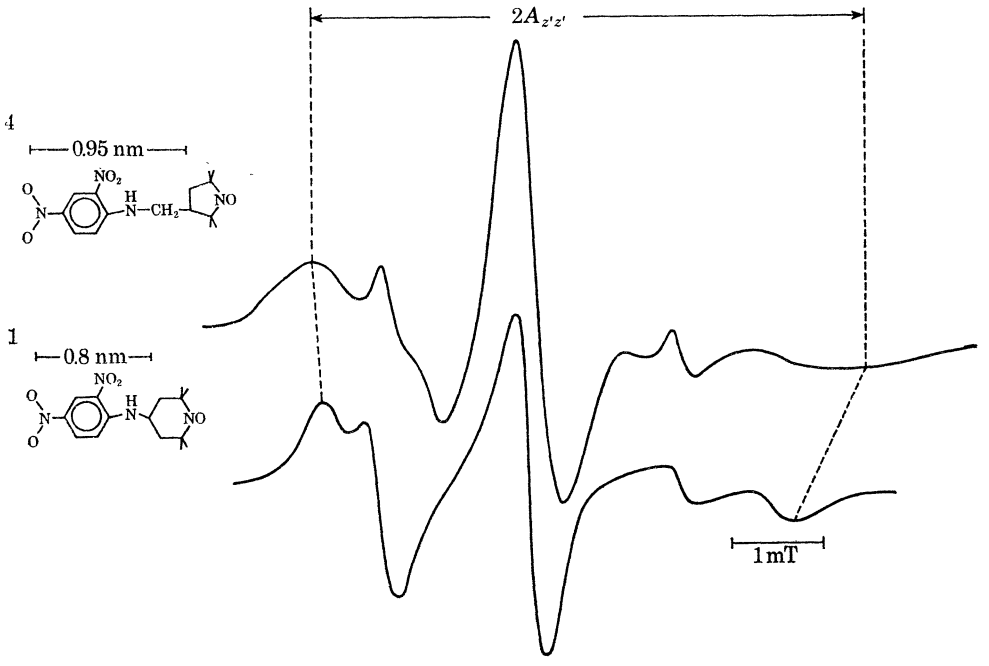

FIGURE 5. E.s.r. spectra of the short five-membered and six-membered ring hapten spin labels bound to the Fv from MOPC 315. (Conditions as in figure 3.)

The extent of motional averaging may be defined by means of an order parameter (Schreier-Mucillo *et al.* 1973, Smith *et al.* 1975)

$$S = \frac{A_{z'z'} - A_{x'x'}}{A_{zz} - A_{xx}}, \tag{1}$$

where $A_{z'z'}$ and $A_{x'x'}$ are defined by the maximum and minimum hyperfine splittings as shown in figure 4 (in some cases the $A_{x'x'}$ splitting cannot be resolved). A_{zz} and A_{xx} are the corresponding values for rigid immobilization and can be determined from the hyperfine splittings obtained with the magnetic field directed along the various nitroxide axes in a single crystal: $A_{zz} = 3.2$ mT, $A_{xx} = A_{yy} = 0.6$ mT (Griffith, Cornell & McConnell 1965; Hubbell & McConnell 1971). The order parameter can be related to the amplitude of spin label motion by (Schreier-Mucillo *et al.* 1973; Smith *et al.* 1975):

$$S = \tfrac{1}{2}(3\langle\cos^2\beta\rangle - 1), \tag{2}$$

where the angular brackets indicate a time average over the instantaneous angular displacement, β.

Table 1. Hyperfine splittings of spin label haptens, bound to the IgA, Fab and Fv from MOPC 315

hapten		$A_{z'z'}$/mT	$A_{x'x'}$/mT	a_0/mT
1	Fv (40 mg/ml)	2.60 ± 0.01	—	—
1	Fv (10 mg/ml)	2.54 ± 0.01	—	—
1	Fab (10 mg/ml)	2.67 ± 0.01	—	—
1	IgA (10 mg/ml)	2.70 ± 0.01	—	—
2	IgA (2 mg/ml)	2.38 ± 0.01	1.13 ± 0.002	1.55 ± 0.01
3	IgA (2 mg/ml)	1.60 ± 0.02	1.60 ± 0.02	1.60 ± 0.02
4	Fab (2 mg/ml)	3.15 ± 1.05	< 1	1.38 ± 0.06†
Free label (aq.)		1.68 ± 0.01	1.68 ± 0.01	1.68 ± 0.01

† Deduced from A_{zz} (max), according to Griffith, Dehlinger & Van (1974).

The hyperfine splittings $A_{z'z'}$ and $A_{x'x'}$ for the various spin labels bound to the different antibody fragments are given in table 1. Ignoring for the moment, the slight differences between the Fv, Fab and IgA fragments, it is clear from a comparison of the six-membered ring hapten labels **1**–**3** that there is a correlation between the hyperfine splitting parameters and the length of the hapten labels. The shorter label, **1**, has the largest value of $A_{z'z'}$, which approaches somewhere near the maximum possible value of 3.2 mT. This together with the rather broad lines in the e.s.r. spectrum show that label **1** is rigidly held in the combining site. The slightly longer spin label hapten, **2**, has a somewhat smaller value of $A_{z'z'}$ and since in this case $A_{x'x'}$ may also be measured (see figure 4), it is possible to use an analysis of the motional averaging of the type embodied in equations (1) and (2) to show that this label has an amplitude of motion of about 50–60° (see Appendix). Examination of molecular models and the analysis in the Appendix shows that this motion could correspond to rotation about just one of the single bonds in the dinitrophenyl-nitroxide linkage. The longer hapten spin label, **3**, is undergoing motion of almost unlimited amplitude, since $A_{z'z'} \approx A_{x'x'}$. However, the motion is still somewhat hindered since the spectral lines are considerably broader than those obtained for the free spin label. These different motional characteristics can be explained by assuming that (*a*) the hapten spin label **1** is accommodated wholly within the combining site, (*b*) the length of hapten spin label **2** is such that the nitroxide ring just protrudes from the combining site and (*c*) that the hapten label **3** is of such a length that the nitroxide ring is just sufficiently clear of the combining site for the protruding section to be able to perform almost complete, angularly isotropic motion (see Appendix). All these considerations would define the length of the combining site as being about 1.1–1.2 nm (Dwek *et al.* 1975).

Analysis of the results with the five-membered ring hapten, **4** (see figure 5 and table 1), enables conclusions to be drawn about both the lateral dimensions and the rigidity of the combining site. The e.s.r. spectrum of hapten **4** has the maximum hyperfine splitting $A_{z'z'} = 3.15$ mT, which together with the very broad lines shows that this label is completely rigidly immobilized in the combining site. This means that the combining site must have considerable structural rigidity, since the hapten can perform no motion relative to the protein molecule. The hapten **1** has a somewhat smaller hyperfine splitting: $A_{z'z'} = 2.6$ mT and slightly narrower lines indicating that, although in this hapten the nitroxide ring is separated by fewer bonds from the dinitrophenyl ring than in the hapten **4**, the spin label must have a limited amount of internal motion which reduces $A_{z'z'}$ from the maximum value of 3.2 mT. Examination of molecular models shows that, because of the different size and orientation of the nitroxide rings in haptens **1** and **4**, their nitroxide groups are situated at similar distances (0.5 nm) from the

dinitrophenyl ring. The spin labels thus both probe the same region of the combining site, and the limited motion of hapten **1** must arise from the increased conformational flexibility in the six-membered ring. This latter point is supported by quantitative analysis of the extent of averaging of $A_{z'z'}$ by transitions between the stable conformers of the six-membered piperidine ring (see Appendix). To accommodate the five-membered ring and the different conformers of the six-membered ring the antibody combining site must have lateral dimensions of approximately 0.9 nm × 0.6 nm (Dwek *et al.* 1975).

(*c*) *Rotational correlation times of the antibody fragments*

Comparison of the $A_{z'z'}$ hyperfine constants for hapten **1** bound to the various antibody fragments (table 1) reveals small differences which depend on the size of the antibody fragments. This small differential averaging of $A_{z'z'}$ is determined by the overall rotational tumbling of the antibody fragment. This view is supported by the fact that $A_{z'z'}$ is increased at higher concentrations of Fv, i.e. with increasing viscosity. A comparative analysis of the rotational correlation times, τ_r, of the protein can be made by determining the extent of averaging of A_{zz} in the Fab and Fv fragments relative to that in the whole IgA, and using the approach of Shimshick & McConnell (1972). (Direct calculation of τ_r for each case is not possible because of the partial A_{zz}-averaging already present arising from the limited motion of hapten **4** relative to the antibody. Hapten **4** which is rigidly immobilized relative to the antibody, is inappropriate for such a study because the very broad lines make it impossible to detect small changes in $A_{z'z'}$.)

As a starting point, the value of τ_r for the whole IgA is approximated by the Stokes–Einstein relation (see, for example, Dwek 1973):

$$\tau_r(\text{Stokes}) = \frac{M\bar{V}}{RT}\eta, \tag{3}$$

where M is the molecular mass, $\bar{V}$ the partial specific volume and η the bulk viscosity. (This treatment makes the assumption that the protein is a rigid sphere of uniform density, and that the microviscosity for its tumbling motion is equal to the bulk viscosity – see, for example, Dwek 1973.) Equation (3) gives a value of $\tau_r = 4.44 \times 10^{-8}$ s for IgA which would correspond to a decrease in A_{zz} of $2\Delta A_{zz} = 0.19$ mT (Shimshick & McConnell 1972). This value is used as a zero-level in the e.s.r. calculation of τ_r for the Fab and Fv fragments. For example, the difference in splitting between Fv (10 mg/ml) and IgA is $2\Delta A_{z'z'} = 0.33$ mT. This value must then be multiplied by a factor of (32/25.4) to correct up the splitting to the value which would be obtained if the hapten were rigidly immobilized relative to the antibody. Addition of the 0.19 mT IgA value to this then gives the result for Fv (10 mg/ml) as $2\Delta A_{zz} = 0.61$ mT, as shown in table 2.

From the values derived from the measured e.s.r. splittings in table 2, it is clear that the τ_r for Fv (10 mg/ml) agrees rather well with the Stokes–Einstein value in keeping with the idea that Fv is a roughly spherical molecule (the Fv (40 mg/ml) value also agrees with the τ_r (Stokes) if the viscosity in this concentrated sample has been increased above the bulk water value to $\eta = 1.5$ mPa s). However the τ_r for Fab is found to be somewhat larger than that predicted by the Stokes rigid sphere value, but this can be correlated with an elongated shape for the molecule. The consistency of the Stokes–Einstein formulation between IgA and Fv for the hapten bound in the same combining site, but with a sixfold increase in the molecular mass of the supporting protein, shows that the differences in the $A_{z'z'}$ hyperfine splitting arise *solely*

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Table 2. Rotational correlation times of IgA, Fab and Fv from MOPC 315

		molecular mass	τ_r (Stokes) 10^{-8} s	$2\Delta A_{zz}$† mT	τ_r (e.s.r.)‡ 10^{-8} s
IgA	(2 mg/ml)	150000	4.44§	0.19	4.44
Fab	(2 mg/ml)	50000	1.48§	0.28	2.3
Fv	(10 mg/ml)	25000	0.74§	0.61	0.65
Fv	(40 mg/ml)	25000	1.1‖	0.43	1.1

† Corrected to rigid immobilization of the spin label, as described in text.
‡ Derived from $2\Delta A_{zz}$ values according to the method of Shimshick & McConnell (1972). For comparison, the value for IgA has been taken equal to the Stokes–Einstein value.
§ Calculated from equation (3) with $\eta = 1$ mPa s, $\bar{V} = 0.72$ cm^{-3} g^{-1}.
‖ Calculated from equation (3) with $\eta = 1.5$ mPa s.

from the rotational tumbling of the whole protein, i.e. that, as viewed by the e.s.r. spectrum of the bound hapten, the combining sites in the whole IgA and the Fv and Fab fragments are identical. Clearly caution must be employed in the interpretation of small differences in hyperfine splittings in cases where such checks cannot be made.

(*d*) *Polarity of the antibody combining site*

The anisotropy of the hyperfine splitting gives a measure of the amplitude of motion as discussed above, but the overall size of the hyperfine splitting constants depends on the polarity of the environment in which the nitroxide is situated (Brière, Lemaire & Rassat 1965; Griffith *et al.* 1974). A convenient index of the environmental polarity is the isotropic hyperfine splitting constant (Smith *et al.* 1975; Griffith *et al.* 1974)

$$a_0 = \tfrac{1}{3}(A_{z'z'} + A_{x'x'} + A_{y'y'}). \quad (4)$$

Where possible, the isotropic splitting constants for the various hapten spin labels have been calculated and are given in table 1. The a_0 value for hapten **4** is deduced from $A_{z'z'}$ (max), assuming the label to be completely immobilized and using the experimental calibration established by Griffith *et al.* (1974).

The hapten label **4** has the lowest value of a_0, corresponding to a hydrophobic environment, suggesting that the five-membered nitroxide ring is bent back into the antibody cavity (as also indicated by the strong immobilization of the label (§3*b*)). This shows that at least part of the combining site is hydrophobic in nature. The hapten label **2** has an anisotropic splitting constant indicative of a region of intermediate polarity, consistent with it being close to the aqueous end of the combining site. Finally the hapten **3** has an a_0 value close to that found for the label in free aqueous solution, confirming that, in this case, the nitroxide is protruding out into the aqueous phase.

It is possible to correlate the spin label polarity measurements with the 'subsites of interaction' of the combining site established by Haselkorn, Friedman, Givol & Pecht (1974) from kinetic binding experiments with various dinitrophenyl haptens. This correlation is illustrated in figure 6, which also summarizes the dimensional data obtained from the anisotropy of the spin label motion. The spatial resolution of the spin label data is not as good as the kinetic mapping because of the limiting size of the nitroxide ring. However, it is clear that the polarity index of hapten label **4**, which is comparable with that of a hydrocarbon solvent, corresponds to the second hydrophobic subsite (S_3) as defined by Haselkorn *et al.* (1974). The

positive subsite (S_4) corresponds to an a_0 in the region 1.55–1.6 mT, i.e. a rather polar environment as might be expected. Correlation of this with the relatively large amplitude of spin label motion in this region suggests that subsite S_4 could quite possibly be at the antibody surface.

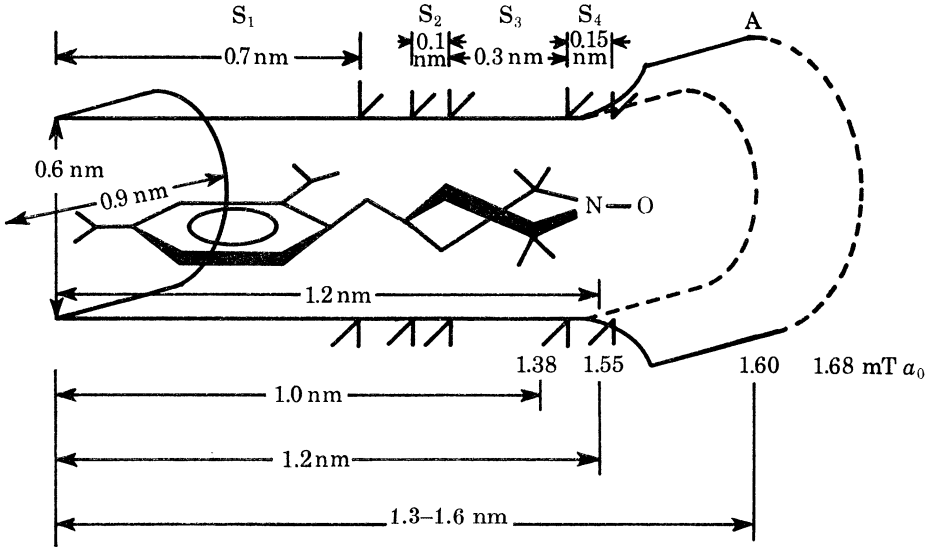

FIGURE 6. Correlation of the polarity measurements of the antibody combining site (deduced from the spin label isotropic splitting factors) with the subsites of interaction deduced from binding kinetics (Haselkorn *et al.* 1974). S_1, 1-*N*-2,4-DNP binding subsite; S_2, first hydrophobic subsite; S_3, second hydrophobic subsite; S_4, positive subsite; A, free aqueous environment. The dimensions of the combining site deduced from the spin label data are also included.

4. AMINO ACID RESIDUES IN THE COMBINING SITE

(*a*) 270 MHz *proton magnetic resonance spectrum of the combining site of Fv from MOPC* 315

The 270 MHz n.m.r. spectra of the IgA, Fab and Fv from MOPC 315 are shown in figure 7. In general the appearance of an n.m.r. spectrum is governed by three factors: the chemical shifts of the various nuclei, the spin-spin coupling constants between nuclei and the width of the resonance. The chemical shifts of most protons occur in a very narrow spectral range and thus in a protein there will be a consequent overlap of many resonances. Also the slow motions associated with proteins of the size of the IgA, Fab and Fv result in relatively broad resonance lines. Since the linewidth may also be greater than the spin-spin coupling constants these too will not be resolved. The overall result is to produce the broad envelopes for n.m.r. spectra such as those shown in figure 7. However, as we expected, we note that the resolution in the spectra becomes progressively better from IgA through Fab to Fv.

Even with Fv however, the problems of resolution and assignment are still major but by a suitable trick this spectrum can be further simplified so that only those resonances in or near to the combining site will be obtained. This trick involves using a paramagnetic centre such as the spin label hapten **1**. Paramagnetic centres such as these cause a broadening of the n.m.r. linewidths. This broadening is *inversely* proportional to the sixth power of the distance between the paramagnetic centre and the nucleus. Thus the most profound effect will be on the resonances of nuclei close to the paramagnetic centre. For illustrative purposes consider an n.m.r. spectrum of four resonances arising from four chemically distinct nuclei (figure 8). Suppose we

now add a paramagnetic spin label to the system which binds very close to one nucleus. The n.m.r. line width of this nucleus is then broadened (figure 8). (For simplicity we shall assume the other nuclei are too far away from the spin label to have their linewidths significantly affected.) If we now subtract the two spectra in figure 8 we shall obtain essentially only the spectrum of the resonances of nuclei close to the spin label. The final spectrum is termed the n.m.r. *paramagnetic difference spectrum.*

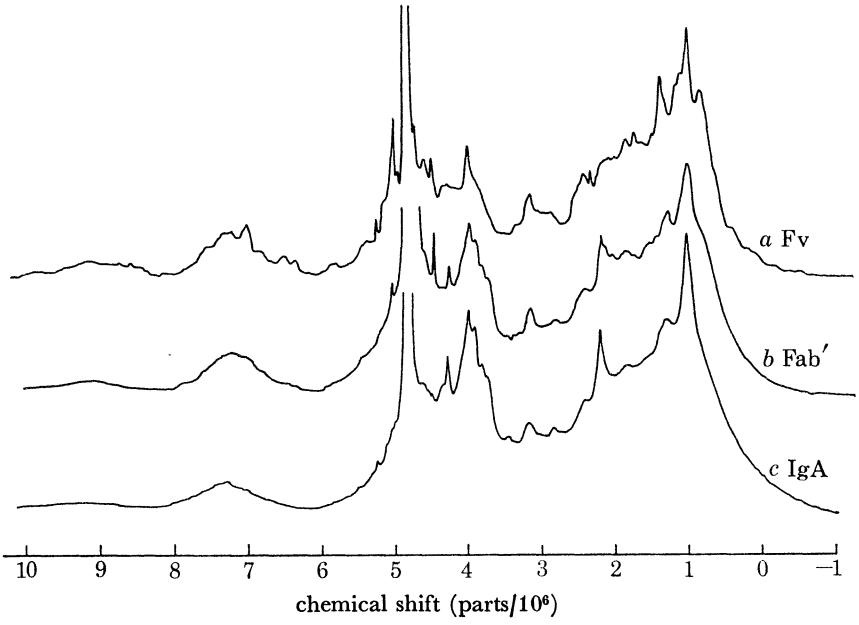

FIGURE 7. 270 MHz proton spectra of Fv, Fab′ and IgA from MOPC 315. (*a*) Fv (30 mg ml^{-1}) 2000 scans; (*b*) Fab′ (40 mg ml^{-1}) 4000 scans; (*c*) IgA (70 mg ml^{-1}) 4000 scans. The IgA sample was run without spinning. (All samples contained 0.15 M NaCl, T = 30 °C, pH (nominal) = 6.9.)

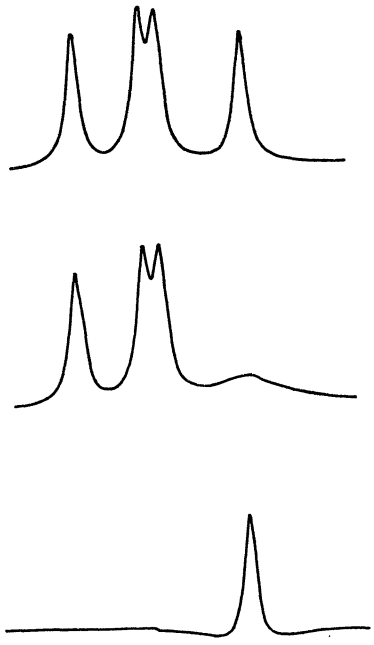

FIGURE 8. Schematic representation of the principle involved in obtaining a paramagnetic difference spectrum.

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Figure 9*a* shows the 270 MHz n.m.r. spectra of the Fv in the presence and absence of the spin labelled hapten **1**. The n.m.r. paramagnetic difference spectrum shows that only about 10% of the n.m.r. spectrum of Fv is affected by the presence of the spin label, and corresponds to the equivalent of about 15 amino acids.

Figure 9*b* shows in more detail two paramagnetic difference spectra at different pHs. We can clearly observe that there are approximately equal numbers of aromatic (6–9 parts/10^6) and aliphatic protons (0–5 parts/10^6) and also that there are slight variations in the paramagnetic difference spectra with pH.

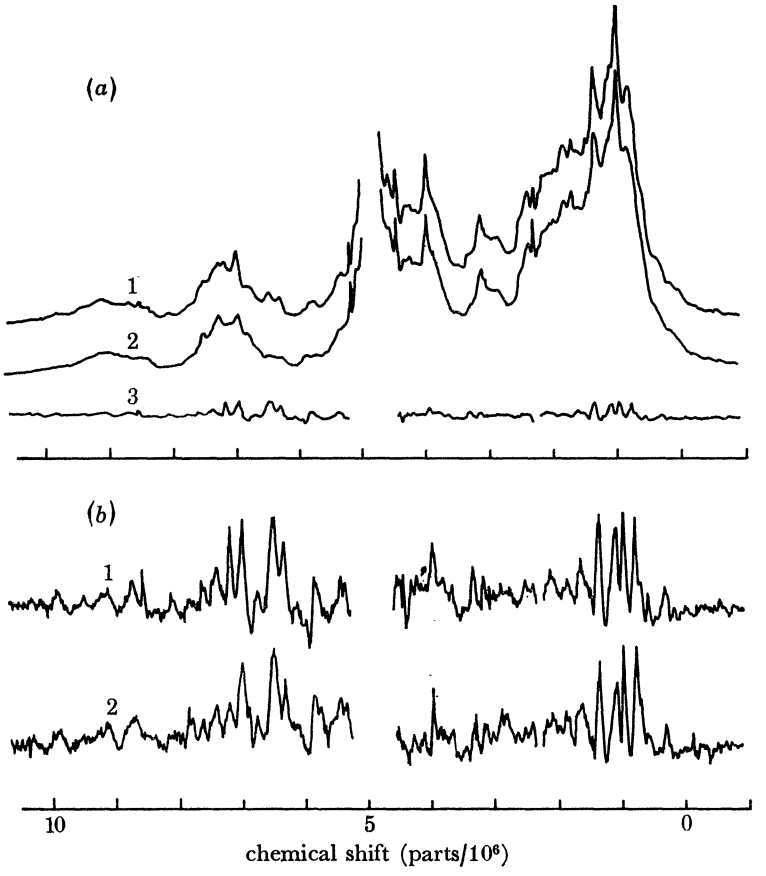

FIGURE 9. (*a*) 270 MHz proton spectrum of Fv fragment from MOPC 315 (30 mg ml^{-1}) T = 30 °C, pH = 6.29, NaCl = 0.15 M, 2000 scans. (1) No addition, (2) in the presence of an equivalent amount of spin label **1**, (3) paramagnetic difference spectrum (1)–(2).
(*b*) Enlarged (×6) paramagnetic difference spectra at (1) pH = 6.9, (2) pH = 8.47. Other conditions as for figure 5 (*a*).

A closer examination of the paramagnetic difference spectra reveals that some of the resonance peaks appear not as a result of broadening by the hapten but rather as a result of chemical shift changes caused by the presence of the hapten. Clearly any such differences arising from changes in chemical shift in the spectra in figure 9 must come from resonances outside the broadening range of the spin labelled hapten. This range has been estimated (Dwek *et al.* 1975) to be over a sphere of radius *ca.* 1.4 nm from the nitroxide moiety of the spin label.

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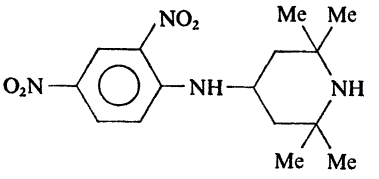
That the presence of the hapten does result in chemical shift changes is illustrated by using the diamagnetic analogue of **1**, namely:



The n.m.r. hapten difference spectrum of this is shown in figure 10*b*. These changes in chemical shift most probably result from conformational changes on hapten binding. These are most

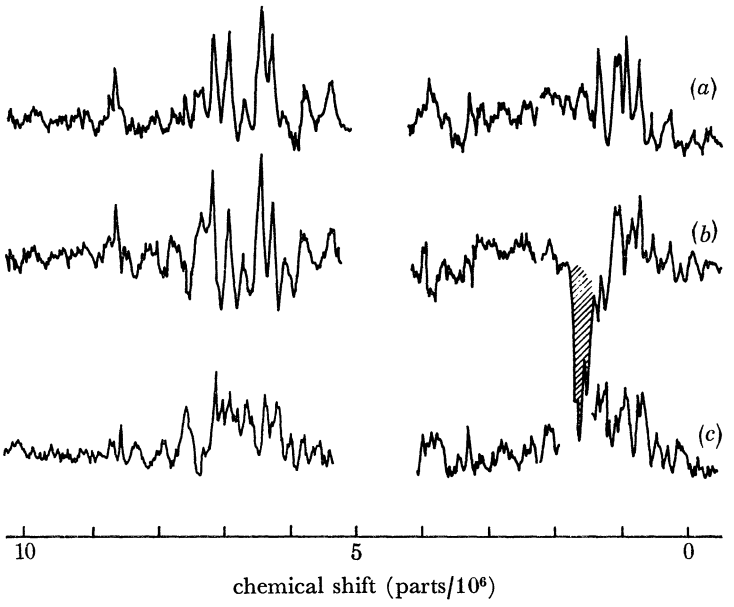


FIGURE 10. 270 MHz proton difference spectra of Fv from MOPC 315. (*a*) Paramagnetic difference spectrum using spin label **1**; (*b*) hapten difference spectrum using the diamagnetic analogue of **1** – i.e. **5**. The shaded methyl resonances originate from the hapten; (*c*) paramagnetic different spectrum using diamagnetic hapten on the control. (Equivalent to (*b*)–(*a*).) The shaded resonances are omitted in this case. pH = 5.80 for (*a*), (*b*) and (*c*). Other conditions analogous to those for figure 9.

probably localized to the binding site in the main since a comparison of figure 10*a* and *b* show that the majority of resonances which occur in the (diamagnetic) hapten difference spectrum as a result of chemical shift changes are also broadened by the spin label. (The large negative peaks in the methyl region (*ca.* 1.5 parts/10^6) in figure 10*b* arise from the methyl resonances of the hapten. The methyl resonances of the spin label hapten itself are, of course, broadened out by the nitroxide grouping as these do not appear in the difference spectrum.)

Figure 10*c* shows the 270 MHz paramagnetic difference spectrum of Fv obtained by subtracting the spectra of (Fv + spin labelled hapten **1**) from that of (Fv + diamagnetic hapten analogue of **1**). This spectrum should contain only those resonances broadened by and therefore near to the spin label moiety.

(*b*) *The assignment of resonances in the combining site*

Having obtained the n.m.r. spectrum of the combining site, the next stage is the assignment of these. This generally involves a variety of intrinsic and extrinsic perturbations (Dwek 1973).

For instance, changing the pH is often used to help identify histidine residues since the chemical shift of the histidine resonances varies with the degree of protonation of the residue. The changes in the n.m.r. spectrum are again most easily followed by the use of difference spectroscopy involving the subtraction of spectra at different pHs. Only those resonances which alter their position with pH will appear in a difference spectrum. Typical results caused by changing the pH on the aromatic resonances of Fv are shown in figure 11*a*. (In this case the diamagnetic

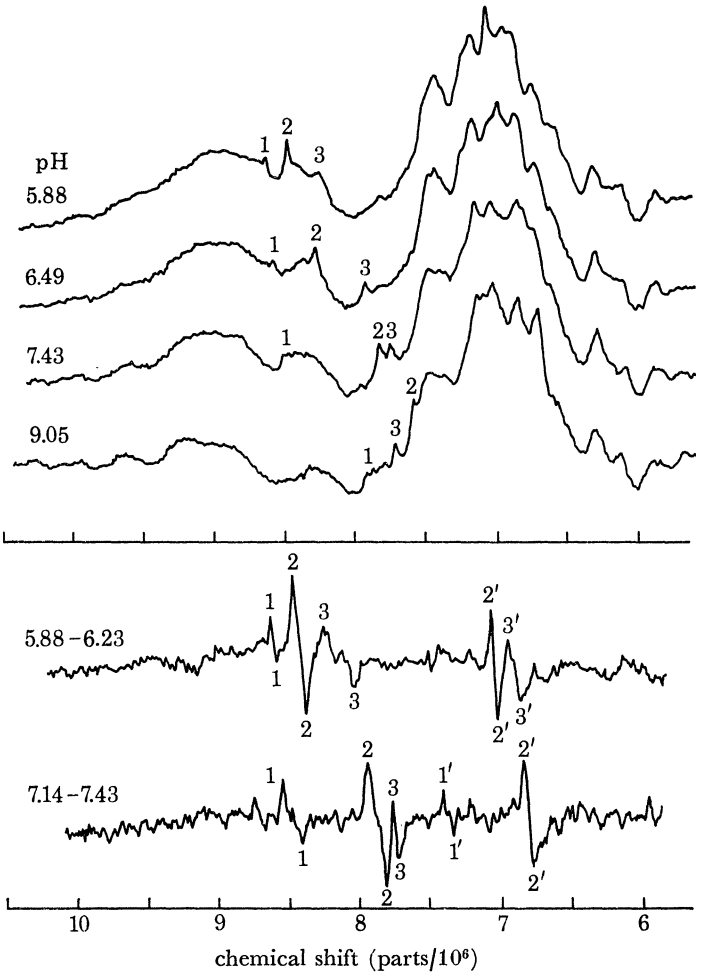


FIGURE 11. (*a*) 270 MHz proton spectrum of the aromatic region of Fv from MOPC 315, in the presence of hapten **5** at three different pH values. The numbers indicate the C2 protons of three titrating histidines. Other conditions as for figure 9.

(*b*) Illustration of pH difference spectroscopy showing both C2 and C4 protons of titrating histidine residues.

hapten is present.) Representative difference spectra are given in figure 11*b*. The results can be presented in the form of a pH titration in which the chemical shifts are plotted against the pH of the solution. Figure 12 shows the results of such titrations for Fv alone and also in the presence of three different DNP-derivatives. The resonances 1, 2 and 3 can be assigned to the C2 protons of the histidine rings and 1′, 2′ and 3′ to the C4 histidine protons (Dwek *et al.* 1975). The dashed numbering is assigned on the basis of the pK_a values which are about 8.1 (resonances 1 and 1′), 6.9 (resonances 2 and 2′) and 6.1 (resonances 3 and 3′).

Resonances 1 and 1′ are anomalous in that they each split into at least two components over

the titration range making their observation rather more difficult than for the other resonances. For this reason the titration curves for resonances 1 and 1′ represent the average of the position of the various components. Within this average we note, from figure 12, that these resonances are insensitive to the presence of the haptens. This is in contrast to those of the other two histidines which suggest that these may be in the combining site.

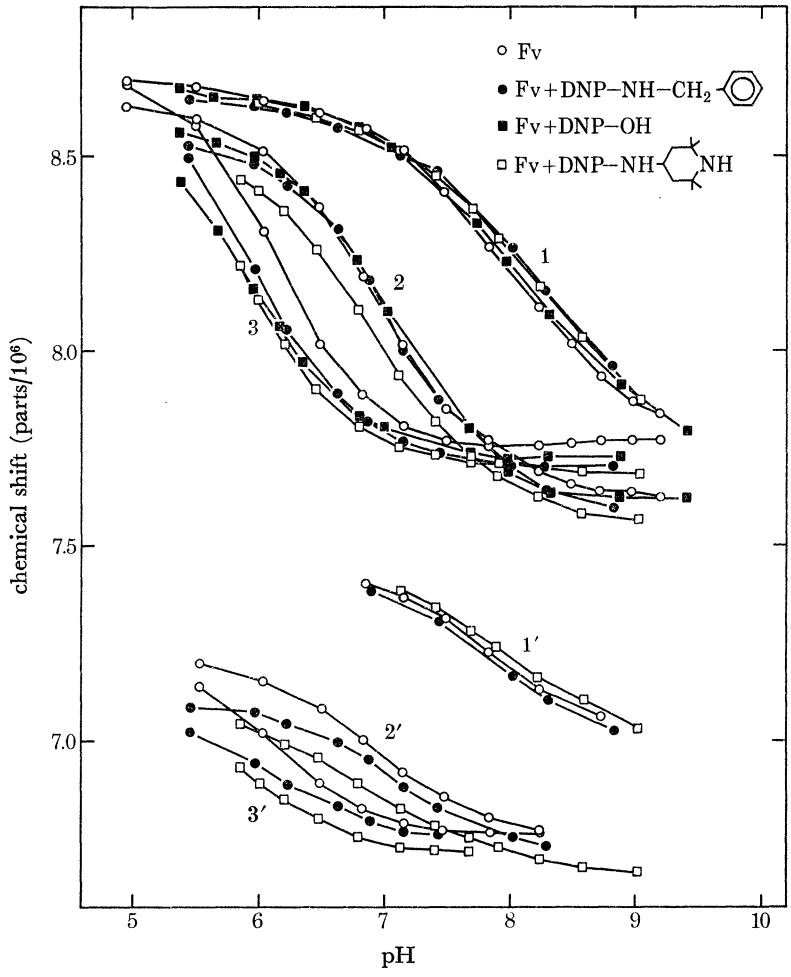


FIGURE 12. Histidine pH titration for the Fv from MOPC 315 above and in the presence of three different haptens.

This conclusion is confirmed by performing similar pH titrations in the presence of the spin labelled hapten **1**. In this case resonances from histidine residues 2 and 3 can no longer be observed over the same pH range. However their resonances do, of course, appear in the paramagnetic difference spectrum. The slight changes with pH observed in figure 9*b* can now be interpreted as arising from the consequent changes in chemical shift of the histidine protons.

From sequence studies it is known that there are three histidine residues in the Fv fragment (Hochman, Inbar & Givol 1973) at positions 44 and 97 in the light chain (Schulenburg Dugas Bradshaw, Simms & Eisen 1973) and at position 102 in the heavy chain (Francis, Leslie, Hood & Eisen 1974). By analogy with human Ig variable region sequences (Kabat & Wu 1971). positions 97_L and 102_H may be presumed to be in hypervariable regions. These latter two positions would also be in the binding site described by Poljak *et al.* (1973) and Amzel *et al.* (1974) in the three dimensional crystal structure of Fab′ New. Histidine 44_L is not in a

hypervariable region nor according to Poljak *et al.* (1973) is it one of the residues in the cleft that forms the combining site for Fab′ New. We would therefore propose that the histidine resonances 2 and 3 arise from His 97_L and His 102_H or vice versa.

The assignment of other resonances to types of amino acids and then specific residues will most probably involve chemical modifications studies on the Fv. However, the identification of the histidine resonances permits a reasonably accurate estimation of the number of protons in the paramagnetic difference spectrum to be assigned. The aromatic region corresponds to the equivalent of about 30 protons and the aliphatic region to 21 protons.

(*c*) *The possibility of geometrical orientations of the amino acid residues in the combining site*

The fact that a particular nuclear resonance appears in the spin label paramagnetic difference spectrum only places that nucleus on a sphere of given radius (in this case about 1.4 nm) from the spin label (nitroxide) group. The geometrical orientation of the nucleus will have to be obtained from measurements of the induced chemical shifts caused by the lanthanides. The induced shift of a nuclear resonance depends on the distance of that nucleus from the lanthanide and also two angles (Dwek 1973). Thus the next stage is to check for intrinsic lanthanide binding sites.

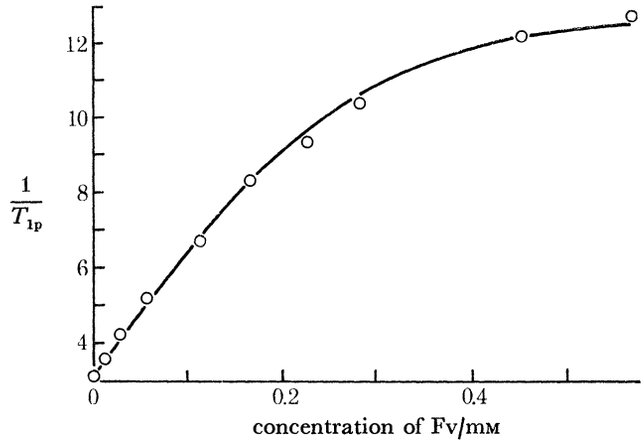


FIGURE 13. The binding of Gd III to Fv from MOPC 315 as monitored by the change in proton relaxation rate of the solvent water ($1/T_{1p}$) at 20 MHz. Conditions: [Gd III] = 274 μM, pH = 5.5, [KCl] = 0.15 M, T = 19 °C.

5. LANTHANIDE BINDING STUDIES

We have used three different techniques to monitor lanthanide binding to Fv from MOPC 315, both in the presence and absence of haptens. The first involves measuring the water relaxation rates in solutions of Fv containing Gd III. The theory of this technique has been described elsewhere (Dwek 1973). Figure 13 shows a titration of a fixed amount of Gd III as a function of Fv concentration at pH 5.5. The titration was followed by measuring the changes in the solvent spin-lattice water relaxation rate as the Gd III is bound to the protein. This and similar titrations at fixed amounts of Fv as a function of Gd III concentration have shown that there is one tight Gd III binding site with a dissociation constant K_D = 30 μM and a weaker site (or sites) (Dwek *et al.* 1975).

The second technique involves measuring the tryptophan fluorescence of Fv (excitation at 295 nm, emission at 340 nm) which is sensitive to the binding of both lanthanide ions and

haptens. The advantages of this method are that the binding of different lanthanides may be readily compared and also reciprocal effects between lanthanide and hapten binding can be elucidated. Titrations with Gd III at pH 5.5 again show that there is one tight metal binding site (K_D = 80 μM). In the presence of spin label hapten **1** the binding of lanthanides as detected by fluorescence is weakened 10–20 fold, with a reciprocal effect on the hapten binding.

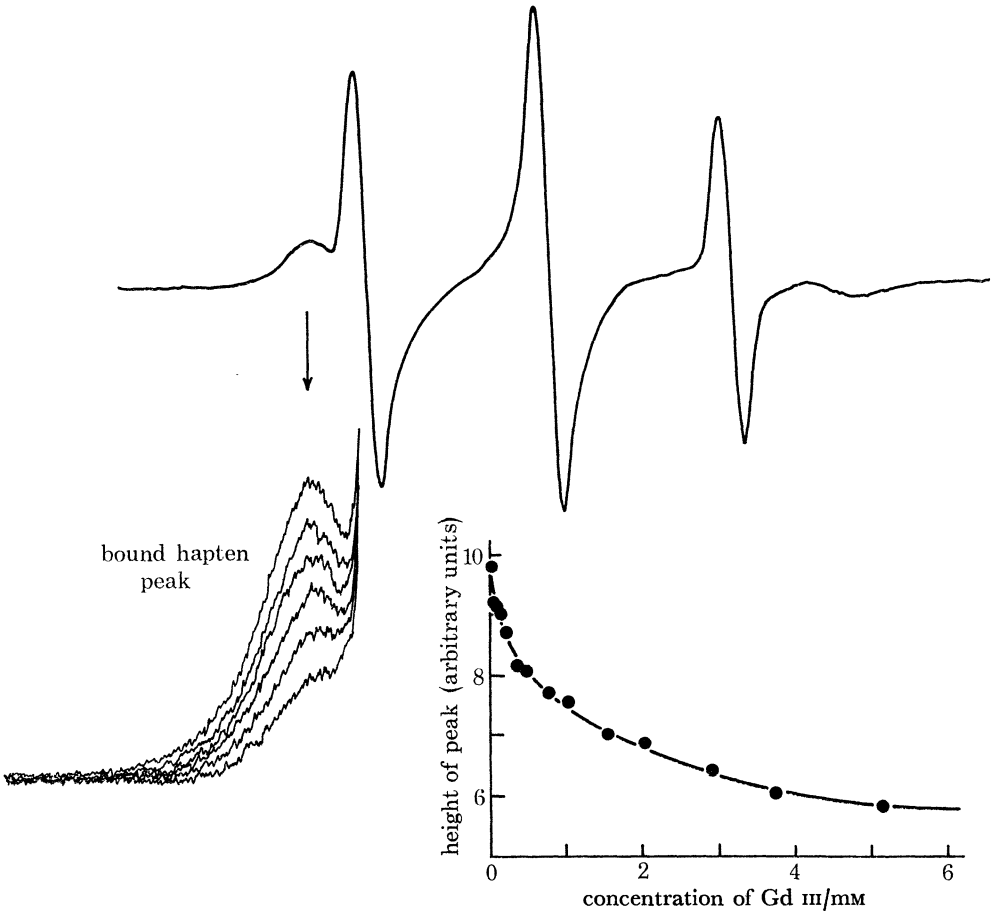


FIGURE 14. The effect of Gd III on the e.s.r. spectrum of the bound spin label hapten **1**. [Fv] = 35 μM, [hapten total] = 33 μM, [NaCl] = 0.15 M, pH = 5.7, T = 20 °C.

The third technique involves measuring the decrease in height of the e.s.r. signal from spin label hapten (**1**) bound to Fv as lanthanides are added, and is capable of providing information on the structural relation between the lanthanide binding sites and the hapten combining site. With diamagnetic La III there is a small decrease in height which may arise from the partial displacement of the spin labelled hapten from Fv, as the metal binds. However with Gd III there is a much larger decrease (figure 14). The extra quenching of the signal height results from the dipolar interactions between the two paramagnetic centres (Dwek 1973), i.e. Gd III and the spin label. Analysis of the resulting Gd III titration curves again suggests that a binding site is present with dissociation constant in agreement with that determined by fluorescence in the presence of hapten. The limiting quenching allows a preliminary estimate of 1.5 nm to be made for this Gd III – nitroxide distance.

TABLE 3. THE BINDING OF LANTHANIDES TO Fv FROM MOPC 315 IN THE ABSENCE AND PRESENCE OF SPIN LABELLED HAPTEN **1** (pH 5.5, 0.15 M NaCl)

metal	spin label hapten **1**	K_D/mM	method used
Gd III	absent	0.03	water relaxation
Gd III	absent	0.08	fluorescence
La III	absent	~0.30	fluorescence
Gd III	present	~2.0	e.s.r.
Gd III	present	~2.0	fluorescence

The results for lanthanide binding at pH 5.5 using the three techniques are summarized in table 3. Preliminary fluorescence and e.s.r. studies indicate that the binding is strongly pH-sensitive so that if magnetic resonance studies under different conditions are required further detailed binding studies would have to be carried out.

In summary, these techniques have shown that at pH 5.5 there is one tight binding site for Gd III and presumably therefore all the lanthanides. The reciprocal effects of weakening of lanthanide and hapten binding strongly suggest that the two binding sites are in close proximity. This is supported by the estimate of 1.5 nm for the Gd III-*nitroxide* distance, but it should be noted that the distance of the lanthanide from the *DNP moiety* in the combining site could be a good deal less than this because of the length of the spin label hapten. This postulate seems reasonable since the major contribution to the free energy of DNP-hapten binding arises from the DNP moiety and the resulting loss in free energy on hapten binding, in the presence of the lanthanide, suggests that it may be the region around the DNP site which is perturbed. The possibility of using lanthanides for the n.m.r. studies are now apparent.

We wish to thank Professors R. R. Porter, F.R.S., D. C. Phillips, F.R.S., R. J. P. Williams, F.R.S. and D. Givol for their encouragement and interest in this work. Dr Israel Pecht kindly sent us the sample of DNP-benzylamine. We also thank the Medical Research Council and the Science Research Council for their support. R.A.D. thanks the Royal Society for the award of a Locke Fellowship. A.C.M. is a recipient of a post doctoral fellowship from the Canadian Medical Research Council. D.M. is an I.C.I. Fellow.

We thank J.E.O.L. Co. Ltd for the loan of an e.s.r. spectrometer.

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Appendix. Calculation of hyperfine structure averaging in the spin label hapten e.s.r. spectrum, for various motional models

Two types of motion are considered: the averaging from conformational transitions within the nitroxide six-membered ring, and the averaging from rotations about bonds joining the nitroxide ring to the anchored DNP group. There are two types of stable conformer for the six-membered nitroxide ring. The first, the so-called 'twisted' conformations (see figure A1), might be expected to be of lowest energy on the grounds of minimum intramolecular steric hindrance between the tertiary methyl groups. The second type of conformers are the boat and chair conformers (see figure A2) which will also be important, and might be favoured for the bound hapten because of intermolecular steric considerations.

(*a*) *The twisted conformations*

Transitions between the two conformers I and II produce an oscillation of the x and z axes of the nitroxide group through $\pm 25°$ (Rozantsev 1970), as indicated in figure A1. Since the conformational transitions are rapid, the axis of motional averaging will be the mean between the two conformations, and the resultant hyperfine constants will be (Smith *et al.* 1975):

$$A^2_{x'x'} = A^2_{xx}\cos^2\theta + A^2_{zz}\sin^2\theta, \tag{A1}$$

$$A^2_{z'z'} = A^2_{xx}\sin^2\theta + A^2_{zz}\cos^2\theta. \tag{A2}$$

Taking $\theta = 25°$, and using the single crystal values: $A_{xx} = A_{yy} = 0.6$ mT, $A_{zz} = 3.2$mT, one gets: $A_{x'x'} = 1.46$ mT, $A_{z'z'} = 2.91$ mT. The value for $A_{z'z'}$, the maximum hyperfine splitting, is reasonably close to the experimental value for spin label hapten **1** of 2.6–2.7 mT. It will be noted from the spectrum of **1** in figure 4, that lines do also occur with an apparent hyperfine splitting in the region of 1.5 mT, but detailed spectral simulation would be required to confirm this; only the maximum hyperfine splitting can be measured reliably. In fact the

motion is a little more complicated than is clear from figure A1, because the conformational transitions also involve an angular oscillation in the y–z direction which would serve to decrease $A_{z'z'}$ even further.

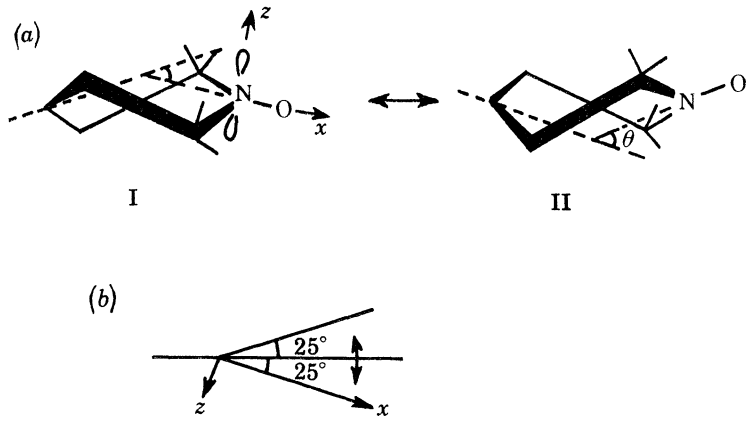

FIGURE A1. (*a*) 'Twisted' conformers of the six-membered nitroxide ring.
(*b*) Rotational averaging of nitroxide hyperfine constants by conformational transitions between conformers I and II, assuming the ring to be rigidly anchored.

(*b*) *Boat–chair transitions*

The only boat and chair conformations which would not involve large lateral displacements of the nitroxide group are those indicated in figure A2. Transitions between these two conformations leads to a displacement of the x and z axes by $\pm 54°$ (see figure A2*b*), and in this case there is no angular displacement in the y–z direction. Application of equations (A1) and (A2) yields the following results: $A_{x'x'} = 2.61$ mT and $A_{z'z'} = 1.94$ mT. Again the maximum splitting (2.61 mT) is close to the measured value of 2.6–2.7 mT, but in this case the motional averaging is such that the axis with the maximum splitting is interchanged. (It is not possible to differentiate between the axes in the e.s.r. spectrum unless a single crystal specimen is available.)

It is difficult to say *a priori* which of the sets of conformers in sections (*a*) and (*b*) will be the more important; if both are present their effects will be proportional to their relative probabilities of occurrence. However, it is clear that conformational transitions between the stable conformers of the six-membered ring are capable of giving rise to the degree of motional averaging observed in the maximum hyperfine splitting of the hapten spin label **1**.

(*c*) *Bond rotation*

Rotation about one of the bonds linking the nitroxide ring to the DNP ring will provide further motional averaging which will be superimposed on that considered above. This is illustrated in figure A2*a* for free rotation about the bond directly attached to the nitroxide ring. In the case of the boat and chair conformers, the geometry of the attachment is such that the x-axes precesses at a fixed angle of 54° to the rotational axis (figure A2*c*), hence $A_{x'x'}$ remains at the value calculated in (*b*) of 2.61 mT. The rotational motion averages the anisotropy in the z–y plane giving an axially symmetric splitting which is the average of $A_{z'z'} = 1.94$ mT (from (*b*)) and $A_{yy} = 0.6$ mT, i.e. the axial splitting value is 1.27 mT. These hyperfine constants of 2.61 mT and 1.27 mT are to be compared with the experimentally measured values of

2.38 mT and 1.13 mT for hapten spin label **2**. The agreement is quite good, indicating that such a rotational motion can provide the necessary averaging. The really significant result, however, is that full 360°-rotation of the nitroxide ring as a whole is required to provide the

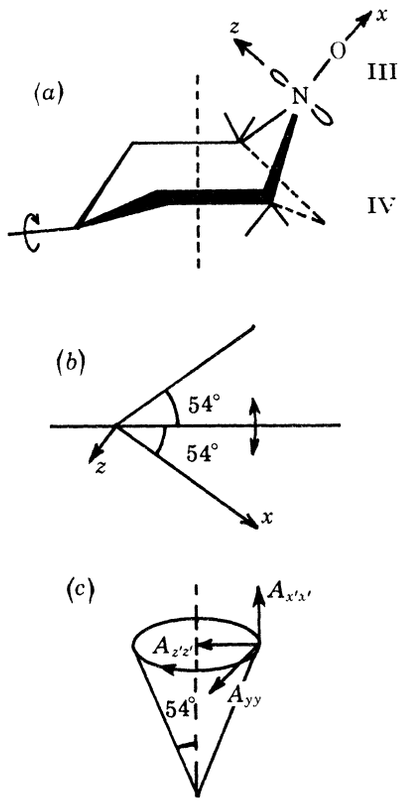

FIGURE A2. (*a*) Chair, III, and boat, IV, conformations of the six-membered nitroxide ring. Conformational transitions are not possible in the left-hand half of the ring because it is anchored to the DNP ring.
(*b*) Rotational averaging of the nitroxide hyperfine constants by conformational transitions between conformers III and IV, assuming the ring to be rigidly anchored.
(*c*) Motional averaging by rotation about the single bond of attachment, as shown in (*a*).

necessary axial averaging giving rise to a well-resolved minimum splitting as seen for hapten **2** in figure 4. This means that the methyl groups of the nitroxide ring must be clear of the antibody cleft, which makes it possible to put a limit on the length of the antibody combining site of 1.1–1.2 nm.

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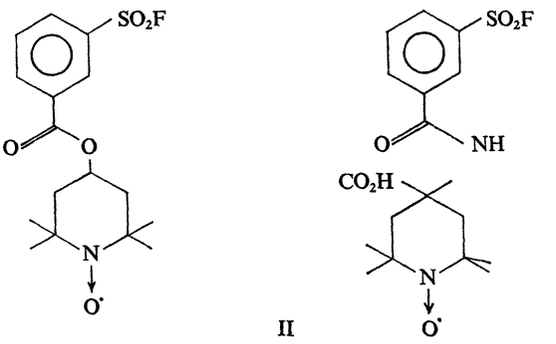
Discussion

L. J. DUNNE (*Department of Physics, Guy's Hospital Medical School, London Bridge, London SE*1 9*RT*)

What method have you used to interpret the e.s.r. spectra? I ask this since I am a little worried about the use of spin labelled haptens to obtain information about the depth of the combining site since the method appears to have no rigorous basis. Particularly, what is the justification for comparing five-ring and six-ring spin labels since in each case the line shape will be partly governed by rapid anisotropic twisting of the nitroxide moiety which will in turn

be governed by the intramolecular interactions involved at the site which will be highly specific and characteristic of each spin label?

One such example of highly specific intermolecular interactions giving anomalous spectra are the spin-labelled inhibitors for α-chymotripsin.



Inhibitor I gives a weakly immobilized spectrum whereas II was highly constrained which was surprising in view of the similar distances involved (see Smith, I. C. P. 1972 *Biological applications of esr* (ed. H. M. Schwartz, J. R. Bolton & D. C. Borg), p. 505 New York: Wiley).

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The method used to interpret the e.s.r. spectra consists of an analysis of the spin label hyperfine splittings in terms of the anisotropic motion of the nitroxide moiety (see table 1). This spin label motion is limited by the geometry of the combining site, and hence constitutes a probe of the binding site dimensions. (This latter point is quite clear, since all the spin labels give rise to a completely isotropic spectrum in free solution, see figure 4.) Since the five- and six-membered ring haptens **1** and **4** have different stereochemical orientations of the nitroxide ring relative to the DNP ring, the allowed bond rotations and conformational transitions for these two hapten labels will be different when they are bound in the combining site; this is observed in figure 5. In fact, the five-membered hapten **4** is totally immobilized, and it is argued in Appendix I (§§ *a* and *b*) that the only allowed motion of the six-membered hapten **1** consists of conformational transitions within the six-membered ring. Both these observations are consistent with the motion of the two hapten labels being limited by a combining site of lateral dimensions 0.9 nm $\times$ 0.6 nm. In this connection it is interesting to note that, in collaboration with Professor D. Givol of the Weizmann Institute, we have recently examined a different DNP-binding myeloma protein, XRPC 25, and in this case both the five- and six-membered haptens **1**, **4** are totally immobilized, indicating that the lateral dimensions of the combining site are narrower in this latter immunoglobulin.

Regarding the sulphonyl fluoride inhibitors of α-chymotrypsin: using reasoning analogous to that used with the spin labelled haptens, Berliner inferred from the spectrum of inhibitor **1** that the binding site was small – sufficient only to accommodate the aromatic ring of the inhibitor. The inhibitor **2** contains several different functional groups which might interact intramolecularly, or with other regions of the enzyme surface, hence giving rise to an immobilized spectrum. For the spin labelled haptens this is not the case, in particular no free carboxyl groups are present, and comparison of the spectra of several different haptens (not just two) give rise to consistent conclusions regarding the dimensions of the combining site.