ions with three-, four-, and five-membered rings undergo ring opening after ca. 10^{-9} s to form initially 1-alkene molecular ions which, depending on the internal energy and ion lifetime, isomerize to some extent to a mixture of double bond isomers. From the identity of the CA spectra (reflecting to a large extent the stable ions) it can be concluded that the energy barrier for such ring opening processes is considerably smaller than the lowest threshold for decomposition and cannot exceed a few tenths of an electron volt.[30]

In contrast, cycloalkane molecular ions with six-, seven-, and eight-membered rings are stable *prior* to decomposition over the entire range of lifetimes and internal energies available in an electron impact mass spectrometer. As already pointed out by Meyerson et al.[4] for methylcyclopentane and methylcyclohexane the difference in the isomerization behavior is most simply accounted for as resulting from the difference in ring strain.

Furthermore the present results rule out that three- or four-membered cycloalkane molecular ions are formed by 1,3 or 1,4 elimination of HX from compounds of the general type RX (X = OH, F, Cl) as has been assumed sometimes in the past[31] which does not exclude that these eliminations proceed via transition states with a cycloalkane-like structure.

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Fluorine–Proton Overhauser Effects in Fluorine-Labeled Macromolecular Systems

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Abstract: Calculations of the $^{19}F\{^1H\}$ nuclear Overhauser effect in a system of nuclei corresponding to a *p*-fluorophenyl residue interacting with three linearly arrayed methylene groups have been performed as a function of the molecular correlation time (τ_c) and the distance (r) between the fluorine nucleus and the nearest methylene group. This collection of 11 nuclei is taken as a model for a fluoroaryl group covalently attached or reversibly complexed to a macromolecule, such that protons of the macromolecule can interact with the fluorine nucleus. Numerical results are presented which indicate the extent to which selective saturation of proton resonances of the macromolecule can be used to identify the interacting group; at 94.1 MHz selectivity diminishes for $\tau_c > 10^{-8}$ s and irradiation at any proton frequency (100 MHz) should lead to loss of fluorine signal intensity.

Fluorine-19 chemical shifts and coupling constants are often an order of magnitude larger than these parameters for a hydrogen nucleus in a similar situation and fluorine relaxation rates are potentially more sensitive to molecular environment than those for protons because of the importance of the chemical shift anisotropy mechanism. Coupled with a

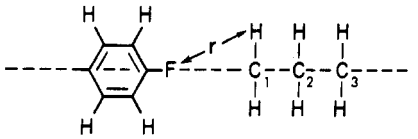

Figure 1. Structure of the 11 spin ½ nuclei model used in the calculations described in the text. The internuclear distances in the left array were those of a *p*-fluorophenyl group. All distances were held fixed for the calculations except *r*.

sensitivity to detection nearly equivalent to ^1H and the observation that a covalently bound fluorine atom is nearly isosteric with covalent hydrogen,[1] these facts have led to a burgeoning interest in the application of fluorine NMR spectroscopy to biochemical systems.[2] These experiments can be classified according to whether the fluorine nucleus is covalently linked to the macromolecule under study[3] or is attached to a small molecule which is in an equilibrium binding situation with the macromolecule.[4] A particularly interesting variant of the first type of experiment is the metabolic incorporation of fluorinated amino acids into proteins, providing multiple fluorine labeled materials for study.[5-7] Consideration of the magnetic resonance properties of the macromolecule-associated form of fluorine nuclei follows the same theoretical principles in both types of experiments but the small molecule binding case is complicated by the presence of uncomplexed small molecules and the necessity to include in the discussion the rates at which equilibrium is maintained.

In several situations it is now clear that interactions between the protons of a protein and an associated fluorine nucleus are responsible for a major fraction of the relaxation rate exhibited by a fluorine nucleus.[8,10] The possibility thus exists that the amino acid of the protein which is involved in relaxing the fluorine can be identified by a selective double resonance experiment in which irradiation at the resonance frequency of interacting proton(s) produces a nuclear Overhauser effect on the intensity of the fluorine signal. Overhauser effects of this type have been observed in cases where protons of the macromolecule are irradiated while protons of a bound small molecule are observed.[11]

In an important series of papers, Hull and Sykes have analyzed in detail the relaxation behavior of fluorine nuclei attached to proteins.[5] They discuss relaxation in a fluorine-labeled protein in terms of three spin systems: (1) the fluorine nucleus, (2) a small number of neighboring ^1H nuclei that directly interact with the fluorine, and (3) all other protons of the protein. Using the nomenclature of Hull and Sykes, nuclei in group 2 will be designated as S spins while those in group 3 will be I spins. There will be intergroup as well as intragroup spin interactions in groups 2 and 3. Depending upon the efficiency of energy exchange between and within groups of spins, these authors note that it is possible that weak single frequency proton irradiation at *any* point in the protein ^1H spectrum may lead to an NOE on the fluorine signal; this is what is observed in the case of fluorine-labeled alkaline phosphatase.[5b] Thus, specificity in the NOE experiment can be lost and little useful information other than the fact that the fluorine nucleus interacts with protons can be obtained. If energy transfer between the I spins and the S nuclei is inefficient, then a specific NOE on the fluorine resonance upon irradiating nuclei of group 2 is possible. Given the experimental observations with proton-proton NOE's, this latter situation must apply in some cases.[11]

The efficiency of spin energy transfer between groups of nuclei depends in an essential way upon the three-dimensional structure of the system under study and the time stability of that structure. Thus, no completely general calculation of spin-lattice relaxation rates or Overhauser effects in fluorine-labeled macromolecules is possible. In this work we have carried out computations using a reasonable model for a fluorine nucleus in a protein environment with the goal of illuminating the extent to which selective $^{19}F\{^1H\}$ NOE experiments can be used to reveal the nature of groups on a protein or other macromolecule which interact directly with the fluorine nucleus.

For our fluorine-containing moiety we choose the *p*-fluorophenyl group. A set of three contiguous methylene groups arranged in a linear fashion as indicated in Figure 1 was included in the model to represent amino acid side chains interacting with the fluorine atom. The use of six protons arrayed in this way represents a rather arbitrary compromise between the seven proton-three carbon side chains of valine and methionine, the six-proton (neglecting OH) system of a tyrosine side chain, and the smaller number of protons in glycine and cystine residues.

The dynamics of motion of the array shown in Figure 1 were described by a single correlation time, τ_c, which shall be taken as characteristic of the tumbling, as a whole, of the (spherical) macromolecule which holds these nuclei. Thus, internal motions of the *p*-fluorophenyl group such as rotation about the C_2 axis or motions of the protein which could modulate the distances between the methylene groups and the fluorophenyl group have been ignored. It is hard to make general statements about the degree to which the neglect of these motions is unrealistic. Planar amino acid side chains in proteins may be found in environments so rigid that the molecular motions mentioned above take place in a time frame that is too slow to influence nuclear relaxation[5a,12,13] and fluorescence and ESR studies of "reporter groups" covalently attached to proteins indicate that a reporter group can be found in situations such that it has no detectable rotational motion of its own independent of the tumbling of the protein.[14] However, there are also many examples of rapid internal motion of aromatic side chains in proteins.[13,15] Our model will therefore likely be valid for only some fraction of real protein systems; calculations which include the effects of macromolecular shape, internal rotation, and the rates of conformational change of the macromolecule are planned and will eventually provide information applicable to the remaining systems.

Methods

Mathematical Formulation. We shall consider a system of loosely coupled ($J/\delta \rightarrow 0$) spin ½ nuclei. Under these conditions relaxation of a given spin by the dipole-dipole mechanism arises from the sum of all possible pairwise interactions in the system; for spin i coupled to the remaining spins j, one can write

$$\frac{d\bar{I}_{z,i}}{dt} = -R_i(\bar{I}_{z,i} - I_{0,i}) - \sum_{j \neq i} \sigma_{ij}(\bar{I}_{z,j} - I_{0,j}) \qquad (1)$$

following the notation of Noggle and Schirmer.[16] Here $\bar{I}_{z,n}$ is proportional to the intensity of the NMR signal from nucleus n, $I_{0,n}$ is the value of $\bar{I}_{z,n}$ at thermal equilibrium, R_i is the total spin-lattice relaxation rate of nucleus i, and σ_{ij} describes the cross-relaxation between spins i and j. The parameter R_i is further broken down as

$$R_i = \sum_{j \neq i} \rho_{ij} + \rho^* \qquad (2)$$

where ρ_{ij} represents the dipole-dipole contribution of each pairwise interaction and ρ^* includes the effects of all other relaxation mechanisms. The only one of these additional mechanisms which will explicitly be taken into account here is the chemical shift anisotropy contribution to relaxation of the fluorine nucleus.[5a] The ρ_{ij} and σ_{ij} are related to the dipole-dipole induced transition probabilities (w_1, w_0, w_2) by

$$\rho_{ij} = 2w_1{}^i + w_0 + w_2 \quad (3a)$$

$$\sigma_{ij} = w_2 - w_0 \quad (3b)$$

For the dipole-dipole mechanism[17]

$$w_1{}^i = {}^3/_{20}g^2J(\omega_i) \quad (4a)$$

$$w_0 = {}^1/_{10}g^2(\cos^2 2\theta)J(\omega_i - \omega_j) \quad (4b)$$

$$w_2 = {}^3/_5 g^2 J(\omega_i + \omega_j) \quad (4c)$$

where $g = \hbar\gamma_i\gamma_j$ and $\cos 2\theta = (\omega_i - \omega_j)/[(\omega_i - \omega_j) + J^2]^{1/2}$. For this work the coupling constant J was set equal to 1 so that the function $\cos 2\theta$ basically was used to discriminate between chemically equivalent ($\omega_i = \omega_j$) and nonequivalent ($\omega_i \neq \omega_j$) nuclei. The spectral densities $J(\omega)$ used were those applicable to isotropic reorientation with a correlation time τ_c[18]

$$J(\omega) = \frac{1}{r_{ij}{}^6}\frac{\tau_c}{1 + \omega^2\tau_c{}^2} \quad (5)$$

Cross-correlation effects have been completely neglected in writing eq 1 and will be assumed negligible. When a particular spin j in the system is saturated $dI_{z,j}/dt = 0$ and $I_{z,j}$ becomes zero in each expression analogous to (1) describing the system. Thus, saturation, in effect, decreases the dimensionality of the mathematical description.

Equation 1 can be cast into the matrix form

$$d\mathbf{I}_z/dt = \mathbf{A} \cdot \mathbf{I}_z - \mathbf{A} \cdot \mathbf{I}_{z,0}$$

where $\mathbf{A}$ is a matrix of coefficients based on the relaxation parameters and $\mathbf{I}_z$ and $\mathbf{I}_{z,0}$ are column matrices of signal intensities at time t and at thermal equilibrium, respectively. A solution to this matrix equation is

$$\mathbf{I}_z = \mathbf{A}^{-1}\mathbf{S}e^{-\Lambda t}\mathbf{S}^{-1}\mathbf{A}(\mathbf{I}_{z,\text{init}} - \mathbf{I}_{z,0}) - \mathbf{A}^{-1}\mathbf{I}_{z,0} \quad (6)$$

where $\mathbf{I}_{z,\text{init}}$ is the initial value for a given magnetization and $\mathbf{S}$ is a transformation matrix which diagonalizes $\mathbf{A}$.[19] The first part of eq 6 shows that the approach of each nucleus of the system to equilibrium (spin-lattice relaxation) will be described by a sum of exponentials, one exponential term for each spin in the system. The last term in (6) represents the Overhauser effect on each nucleus as $t \rightarrow \infty$. The fluorine Overhauser effect in this work is defined as the fractional enhancement

$$f_F(H) = (I_{z,F}{}^{t\rightarrow\infty} - I_{0,F})/I_{0,F} \quad (7)$$

and will range from 0.53 at small correlation times to −1.06 in the slow motion limit if only the dipole-dipole mechanism is considered.

A general program was devised which accepted as input the resonance frequencies and internuclear distances of a system of spin ½ nuclei and then computed the relaxation behavior and the NOE at various correlation times. The chemical shift anisotropy contribution to fluorine relaxation was computed by the prescription of Hull and Sykes.[5a] The thermal equilibrium intensities (magnetization) for each spin were taken to be proportional to the gyromagnetic ratio of the spin[20] and the initial ($t = 0$) magnetization for each spin was computed according to $I_{z,\text{init}} = (\cos\alpha)I_{z,0}$ where α is the angle the sample magnetization is flipped by the initiating pulse(s) of the experiment.

Structural Parameters. The appropriate internuclear distances found in fluorobenzene were for the *p*-fluorophenyl group.[21] The distance (r) between methylene group 1, as indicated in Figure 1, will be varied; internuclear distances within the three methylene fragment will be held fixed as defined in an ideal staggered hydrocarbon[22] ($r_{C\text{-}C}$ = 1.54 Å, $r_{C\text{-}H}$ = 1.10 Å).

An alternative way of viewing the model system is that one or two of the methylene groups represents an amino acid side chain in close proximity to the fluorinated residue (S spins) while the remaining methylene(s) can be regarded as other protein side chains which interact with the first in a less specific manner (I spins). In this context one should note that the distance between methylene groups is about 2.5 Å while consideration of a model of α-chymotrypsin,[23] taken to be a representative protein structure, suggests that the distances between S spin and I spin protons will usually be 4–10 Å.

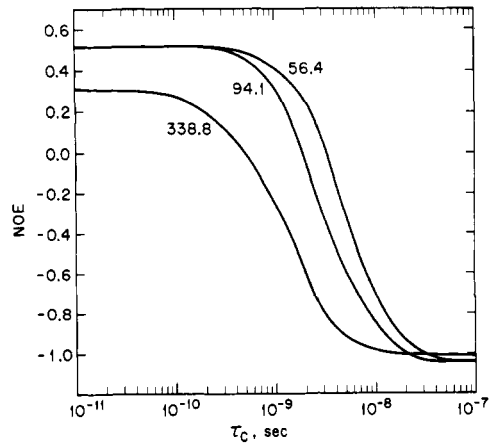

Figure 2. Computed dependence of the $^{19}F\{^1H\}$ nuclear Overhauser effect on the correlation time, τ_c, and radiofrequency when all protons of the model system are irradiated.

Although the model system is a linear array of nuclei and thus not likely to be entirely appropriate to an actual protein, because of the $r_{ij}{}^{-6}$ dependence of the dipole-dipole relaxation effects, the results will be dominated by the nearest neighbors and be less sensitive to smaller changes in other internuclear distances which would arise by departures of the model from a nonlinear configuration.

Results

The fluorine nuclear Overhauser effect is expected to depend upon the correlation time τ_c as well as the radiofrequency used to observe the signal.[16,24] Figure 2 displays the computed $^{19}F\{^1H\}$ NOE for the model system when r = 2.2 Å and all protons are saturated. The attenuation in the magnitude of the effect at 338.8 MHz (protons at 360 MHz) arises from the inclusion of the chemical shift anisotropy mechanism which becomes important at high magnetic fields.[5a] The remaining calculations in this paper were done for a fluorine frequency of 94.1 MHz, although the frequency dependence expressed by Figure 2 is expected to be observed in the situations which follow.

When only methylene group 1 is irradiated, the effects shown in Figure 3 are computed. As expected, the NOE is seen to be dependent on the separation r; at short distances interaction of this methylene with the fluorine strongly dominates the spin-lattice relaxation of this nucleus. At intermediate distances (5–10 Å) which should be typical of many side chain-side chain interactions, the NOE hovers close to zero except at very long correlation times. Various perturbations of the model system were examined with no substantial change in the results shown. In particular, very large ρ^* terms for the protons at each end of the array were introduced to simulate the effect of relaxation provided by additional interactions with groups of the macromolecule. Even with R_1 values of 1000 s^{-1} for these nuclei, no changes in the results summarized in Figure 3 were found.

Figures 4 and 5 record the computed effects of irradiating the second and third methylene groups, respectively. At small correlation times a slight "three-spin" effect[25] is noted on the fluorine resonance upon irradiation of methylene group 2 al-

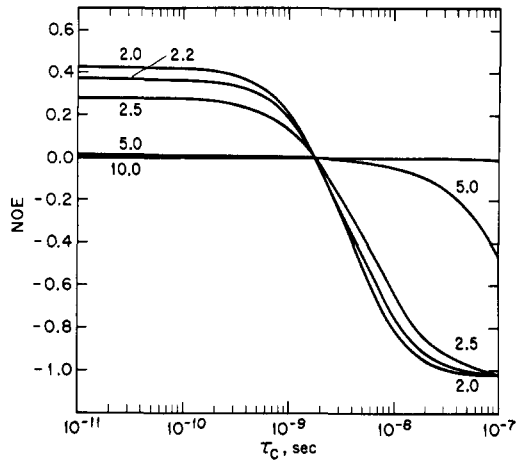

Figure 3. The $^{19}F\{^1H\}$ NOE at various values of the internuclear distance r for the model system when only methylene group 1 is irradiated.

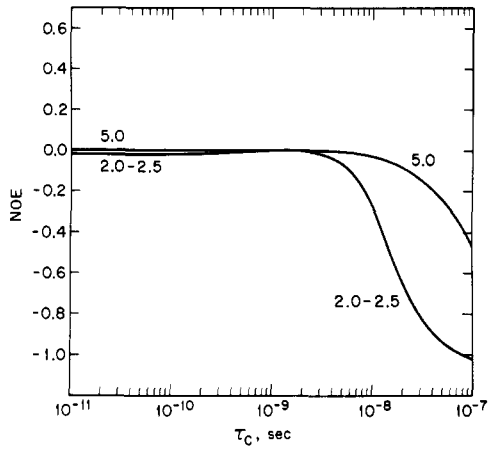

Figure 4. The calculated $^{19}F\{^1H\}$ NOE for the model system when methylene group 2 is selectively irradiated.

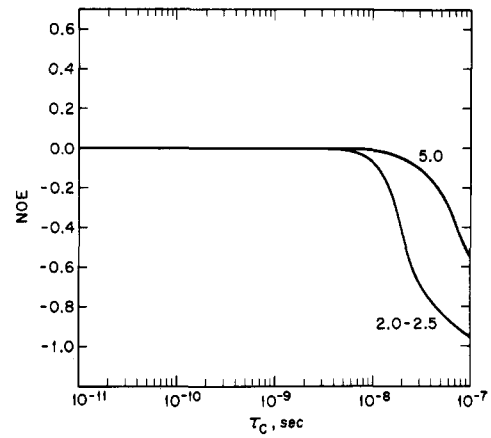

Figure 5. The $^{19}F\{^1H\}$ NOE at various values of r when methylene group 3 is irradiated.

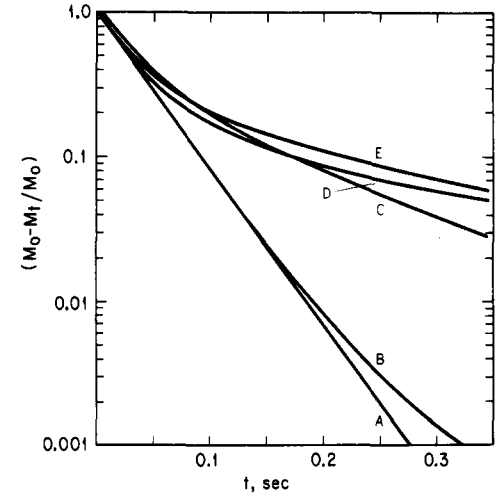

Figure 6. Computed spin-lattice relaxation curves when r = 2.0 Å in the model system and $\tau_c = 1.67 \times 10^{-8}$ s. Curve A is for the fully decoupled system where strictly exponential relaxation is expected while curve E is for the fully coupled situation. Curves B, C, and D represent the computed relaxation behavior upon selective irradiation of methylene groups 1, 2, and 3, respectively.

though it is doubtful that this would be experimentally detectable. Irradiation of methylene group 3 is predicted to produce no NOE until the correlation time becomes long.

With slow molecular motion and short internuclear distances the fluorine spin-lattice relaxation curves for the cases where methylene group 2 or 3 is irradiated are predicted to be strongly nonexponential (Figure 6) while the relaxation curve when methylene group 1 is saturated under these conditions is essentially exponential. A consideration of the degree of nonexponentiality of relaxation curves may thus prove useful in elucidating the stereochemical basis for an observed Overhauser effect. With rapid molecular motions ($\tau_c < 1 \times 10^{-8}$) spin-lattice relaxation is calculated to be exponential at all separations (r) in our model.

When the four aromatic protons of the *p*-fluorophenyl group are irradiated, the Overhauser effects shown in Figure 7 are computed. The total relaxation of the fluorine-19 nucleus is dominated by the protons of methylene group 1 at small r and the magnitude of the fluorine NOE varies according to the fraction of fluorine relaxation provided by the irradiated nuclei.

Discussion

As molecular correlation times become longer the relaxation processes quantified by the rate constant w_0 become relatively more effective in promoting relaxation. Hull and Sykes have noted that this effect could potentially lead to loss of "resolution" in $^{19}F\{^1H\}$ NOE experiments with fluorine-labeled macromolecular systems because irradiation at any proton frequency in the lattice can, through operation of the w_0 processes, lead to saturation of the hydrogen atoms that relax the fluorine nucleus.[5a] The efficiency of w_0 depends strongly upon the internuclear distance as well as the correlation time and our results suggest that it is only when the correlation time becomes longer than $\sim 10/\omega_F$ and the distance between the S and I nuclei becomes shorter than ~2.5 Å will the loss of specificity in the NOE experiment be serious. This can be seen by consideration of Figure 5 if we let methylene group 3 in our model represent the I proton spins of the protein while methylene groups 1 and 2 are taken as the S spins. The distance between the I spins and methylene group 2 is 2.5 Å and is probably shorter than would be a typical I-S distance in a protein. Thus, the limitations to specificity in the $^{19}F\{^1H\}$ NOE experiment suggested by Figure 5 probably represent a worst-case estimate. Using the approximation[26] $\tau_c \approx$ molecular weight $\times 10^{-12}$, one concludes that the selective $^{19}F\{^1H\}$ nuclear Overhauser experiment to identify interacting amino acids in a fluorine-labeled protein should be routinely useful for molecular weights less than about 20 000. With larger macromolecules, the observed effect will depend more critically upon the number of interacting nuclei and the details of their stereochemical relations. For molecules with molecular weight

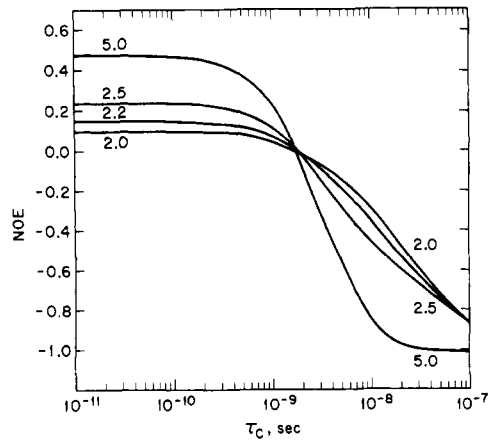

Figure 7. The $^{19}F\{^1H\}$ NOE at various values of the distance r when the aromatic protons are selectively irradiated.

greater than ~100 000 it is doubtful if the $^{19}F\{^1H\}$ NOE experiment will provide useful information unless, as Hull and Sykes have noted, a particular S nucleus has a uniquely distinct resonance which can be saturated or the arrangement of amino acids about the fluorine nucleus is such that I-S interactions are quite ineffective.

The limitations described above are for systems in which the reporter group and its immediate environs reorient with the correlation time characteristic of the overall tumbling of the macromolecule. Rotations or segmental motions of the fluorinated group and the nuclei which interact with it will change the above picture if these motions are more rapid than overall tumbling.[5b]

An experimental aspect of the $^{19}F\{^1H\}$ NOE experiment is worth emphasizing at this point. When irradiating a particular proton resonance, one seeks to drive its equilibrium magnetization as close to zero as possible. At equilibrium, the extent of saturation is given by[27]

$$f = \frac{I_z^{\infty}}{I_0} = \frac{1 + T_2^2(\omega_0 - \omega)^2}{1 + T_2^2(\omega_0 - \omega)^2 + S} \tag{8}$$

with

$$S = \gamma_H^2 H_2^2 T_1 T_2$$

Here T_1 and T_2 are the spin-lattice and transverse relaxation times, respectively, of the proton being irradiated, ω_0 is its Larmor frequency, and ω is the frequency of the H_2 irradiating field. One can arbitrarily define a band, B, of irradiation frequencies such that the fraction f is 0.5 or smaller. Then, from eq 8

$$B = (S - 1)^{1/2} w_{1/2} \tag{9}$$

with $w_{1/2}$ equal to the line width at half-height of the proton signal being saturated. In seeking to minimize f one can increase H_2 to increase the saturation factor, S. However, in doing so, the band width B also increases. To produce 99% saturation of the proton signal would require $S = 100$ but at this level of H_2 power irradiating within a band of frequencies $\sim 10 w_{1/2}$ wide will produce 50% or greater saturation. The resonance line widths $w_{1/2}$ for proteins tend to be 10-30 Hz[28] and thus irradiating over a band of proton frequencies 100-300 Hz wide will produce 50% or more of the maximum NOE on a fluorine signal. (It is interesting that many of the published proton-proton NOE experiments with protein systems show band widths of this order of magnitude.[24,29]) It is clear that in order to obtain optimum resolution in a selective NOE experiment with macromolecules the magnitude of the saturation factor S will have to be carefully considered and controlled.

Our results underline the warning of Hull and Sykes that the interpretation of the $^{19}F\{^1H\}$ NOE experiment with fluorine-labeled macromolecules is not necessarily straightforward.[5a] However, for carefully executed experiments on macromolecules such as small proteins, they should provide useful stereochemical and dynamical information.

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