SOLUTION CONFORMATION OF PEPTIDES BY THE INTRAMOLECULAR NUCLEAR OVERHAUSER EFFECT EXPERIMENT

STUDY OF VALINOMYCIN-K^+

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ABSTRACT This study demonstrates how the intramolecular nuclear Overhauser effect (NOE) experiment can be employed quantitatively to select from a set of possible conformations for a peptide or a protein the particular conformation (or a group of conformations) most consistent with the data. This procedure is demonstrated on a model depsipeptide system—valinomycin K^+ in $CDCl_3$—for which the solution conformation has been inferred by other methods. The NOE enhancements are very sensitive to the conformations assumed by this antibiotic. It is shown that the set of conformations, collectively labeled as A_2 (including the X-ray crystallographic structure) gives a very good description of the NOE enhancements. The structure proposed by Bystrov et al. (1977. *Eur. J. Biochem.* **78**:63) for the uncomplexed valinomycin in nonpolar solvents is also consistent with the experimental data on the potassium complex. Using statistical hypothesis testing involving the Hamilton *R*-factor ratio criterion, all the other models have been rejected as inconsistent with the experimental data. A general formalism is presented for describing the NOE effects in isotropically reorienting molecules. The formalism is not restricted to the extreme narrowing limit of the rotational correlation times and hence applies to both small and large molecules. Some of the factors that can influence the NOE measurements, viz. anisotropic rotational diffusion, conformational averaging, and nuclear spin diffusion, have been considered in this study.

INTRODUCTION

Proton relaxation of organic molecules and biomolecules (especially in nonaqueous, deuterated, and degassed solvents) is usually dominated by intramolecular proton dipole-dipole relaxation (1). Because of the strong dependence of these dipolar relaxation rates on internuclear distance, any experiment that measures these rates or func-

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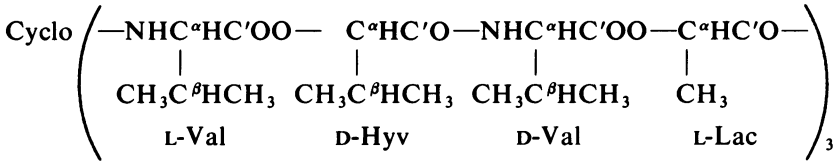
tions of these rates can yield significant information on molecular geometry. The application of one such method, the intramolecular nuclear Overhauser effect (NOE) experiment, to the quantitative evaluation of the structure of small molecules is well documented (2). The NOE experiment measures the steady-state effect of saturation of a specific spin transition or group of such transitions on the intensities of other resonances in the nuclear magnetic resonance (NMR) spectrum. The extreme narrowing approximation ($\omega^2\tau_c^2 \ll 1$, where ω is the resonance frequency and τ_c the rotational correlation time) simplifies the analysis of NOE experiments of most simple organic molecules (1, 2). Failure of proteins and most peptides of even moderate size to satisfy extreme narrowing conditions, particularly at the high resonance frequencies employed for ^{1}H NMR studies of these complex molecules, requires that a more general formalism be developed for analysis of their NOE's. This study is directed, in part, towards this end. In addition, the effects of anisotropic motion, conformational averaging, and spin diffusion on intramolecular NOE's have been considered in this study. While the major emphasis is on peptides, the extension of the formalism to proteins is necessary because of the similar structure of these molecules and the vague and arbitrary distinction between these classes of macromolecules. Consequently, large peptides exhibit many of the characteristics of proteins, particularly when investigated in relatively viscous media commonly employed in NMR studies of peptides (e.g. dimethyl sulfoxide [DMSO]). Our treatment of phenomena such as spin diffusion, already discussed by other investigators (3–7) in the context of its relevance to NMR studies of proteins, is not intended to be comprehensive, but focuses on considerations relevant to the NOE studies of peptides and proteins. Some preliminary attempts at quantitatively applying the NOE technique to peptides have already appeared (8–14), including a semi-quantitative description (11) of the conformation of the antibiotic valinomycin in DMSO.

This paper is directed at a quantitative study of peptide conformations by the intramolecular NOE technique. In particular this study addresses itself to the following question: Given a set of proposed conformations for a peptide, how can one choose one particular conformation (or a group of conformations) that best fits the data? Our procedure consists of first performing a series of NOE experiments involving saturation and observation of both the backbone and side chain protons. Next, for each of the conformations we generate the structure of the peptide using the proposed torsion angles for the backbone (ϕ, ψ) and side chain (χ) orientations and compute the theoretical NOE values for a simulated experiment. Finally, employing statistical hypothesis testing techniques, we compare the goodness of fit of these models to the experimental data.

Choice of the Model Peptide System: Valinomycin-K^+

To establish the utility of the NOE technique in the quantitative analysis of peptide conformations, it is essential first to demonstrate its application to a model peptide for which the molecular geometry has been well characterized by other methods. The dodecadepsipeptide antibiotic valinomycin is one of the more extensively studied pep-

tides and has been characterized by a variety of methods, including X-ray crystallography, infrared spectroscopy, NMR techniques, ultrasonic relaxation, and potential energy calculation (15–23).



Biological interest in valinomycin arises from its ability to sequester K^+ and Rb^+ ions selectively, in preference to Na^+, and to serve as a mobile ionophorous carrier of the former ions across lipid bilayer membranes (24). Valinomycin enhances the cation permeability of membranes of mitochondria (25), erythrocytes (26, 27), and synthetic lipid bilayers (28, 29). The conformation of free valinomycin is strongly solvent-dependent, the general conformational equilibrium being described by the scheme $A \rightleftharpoons B \rightleftharpoons C$, where each of the conformations A, B, and C represents a group of closely related structures (15). The conformations represented by A dominate in nonpolar media, whereas B and C are prominent in solvents of medium and high polarity, respectively. X-ray crystallographic studies (22) of the conformation of valinomycin-K^+ indicate that its structure belongs to the A_2 class of A conformations. The same conformation is preserved in nonpolar solvents. It has been shown that the valinomycin K^+ complex is very stable (23, 30, 31), the backbone being stabilized in a relatively rigid conformation by coordination of the metal ion to all six ester C=O groups, as well as by six intramolecular 4 → 1 hydrogen bonds involving the amide C=O groups. Since the experimental evidence to date indicates that the solution conformation of this complex in nonpolar solvents is consistent with that of a relatively rigid structure, at least for the depsipeptide backbone, we expect that the analysis of NOE experiments will not be complicated by conformational averaging. This molecule exhibits a very simple NMR spectrum with well separated resonances at 270 MHz and shows no evidence of molecular aggregation. Thus, the valinomycin-K^+ complex is an ideal model for demonstrating the quantitative application of the NOE experiments to peptides.

THEORY

The Intramolecular NOE

The equation of motion for the average magnetization $\langle I_{zi} \rangle$ of spin i in a set of N weakly coupled spins is given by a generalization of Solomon's equations (32, 2) as

$$-\frac{d}{dt}\langle I_{zi}\rangle = \sigma_{ii}(\langle I_{zi}\rangle - I_{0i}) + \sum_{j}{}' \sigma_{ij}(\langle I_{zj}\rangle - I_{0j}). \qquad (1)$$

The thermal equilibrium value of $\langle I_{zi} \rangle$ is denoted by I_{0i}. The effect of cross-correlation between the various nuclear spins has been considered negligible (2). The prime on the summation indicates that $j \neq i$. In Eq. 1, the quantities σ_{ij} describe the

various relaxation rates that govern the time evolution of the magnetization of the nuclear spins. These are given by (2, 11)

$$\sigma_{ij} = W_2(ij) - W_0(ij), \qquad i \neq j \tag{2}$$

$$\sigma_{ii} = \sum_{j}' \rho_{ij} + \rho_i^*, \tag{3}$$

and

$$\rho_{ij} = 2W_1(ij) + W_0(ij) + W_2(ij). \tag{4}$$

The quantities $W_0(ij)$, $W_1(ij)$, and $W_2(ij)$ are the transition probabilities, respectively, for the zero-, single-, and double quantum transitions involving the nuclear spins i and j. The parameter ρ_i^* takes into account the self-relaxation of spin i, due to mechanisms like spin-rotation interaction, or effectively self-relaxation processes like dipolar interactions involving paramagnetic impurities or directly bonded ^{14}N. The parameters σ_{ij} $(i \neq j)$ are referred to as cross-relaxation terms that couple the magnetization of spin i with that of spin j. The quantity σ_{ii} is the relaxation rate of spin i that would be obtained as the initial rate (33) in a selective T_1 experiment involving this spin. The NOE observed for spin i, $f_i(S)$, is defined as the fractional intensity change of the resonance of i when a particular spin s or a group of such spins collectively designated as S are saturated, and is given by

$$f_i(S) = (<I_{zi}> - I_{0i})/I_{0i}. \tag{5}$$

Rewriting Eq. 1 under steady state, we obtain through the use of Eq. 5 a set of simultaneous equations for the NOE's, $f_i(S)$, as

$$\sum_{j}'' \sigma_{ij} f_j(S) = \sum_{s} \sigma_{is}, \quad i \neq s, \tag{6}$$

where the double prime on the summation indicates that the saturated spins are to be excluded from the summation. The summation on the right-hand side runs over all the saturated spins. The system of equations (6) given above is quite general and can be solved to obtain the various NOE values $f_i(S)$. We can rewrite Eq. 6 to obtain an expression for $f_i(S)$ as

$$f_i(S) = f_i(S)_{\text{dir}} + f_i(S)_{\text{ind}}, \tag{7}$$

where $f_i(S)_{\text{dir}}$ and $f_i(S)_{\text{ind}}$ represent, respectively, the direct and the indirect contributions to the total NOE. They are given by

$$f_i(S)_{\text{dir}} = \sum_{s} \frac{\sigma_{is}}{\sigma_{ii}}, \quad i \neq s, \tag{8}$$

$$f_i(S)_{\text{ind}} = -\frac{\sum_{j} \sigma_{ij} f_j(S)}{\sigma_{ii}}, \quad j \neq i, s. \tag{9}$$

The $f_i(S)_{ind}$ is also commonly referred to as the three-spin effect contribution (2). If the internuclear distance between i and s is short enough compared to the remaining internuclear distances, then $f_i(S)_{ind}$ usually makes a negligible contribution to $f_i(S)$ for the short correlation limit of molecular motion. For peptides and proteins, however, $f_i(S)_{ind}$ generally cannot be neglected.

Noggle and Schirmer (2) have derived a similar set of equations to describe a multi-spin system that satisfies the extreme narrowing condition ($\omega^2\tau_c^2 \ll 1$, where ω is the Larmor frequency and τ_c is the correlation time). Peptides of even moderate size (mol w $\geq$ 1,000) generally do not satisfy this condition for homonuclear ^{1}H NMR NOE studies (particularly in viscous solvents such as DMSO). The equations derived in this paper and previously (11) are more general and therefore apply not only to small organic molecules but also to peptides and proteins.

In the following discussion we will limit ourselves to a consideration of spin $\frac{1}{2}$ systems. Extension to higher spin cases is straightforward (1), but is of less practical interest. We will also examine some of the factors that can influence the NOE enhancements in peptides and proteins and hence should be considered in a general NOE experiment.

Dipole-Dipole Interactions in the Limit of Isotropic Molecular Reorientation

In this limit, all the internuclear vectors $\mathbf{r}_{ij}$ in the molecule will have the same rotational correlation time. The transition probabilities $W_2(ij)$, $W_1(ij)$, and $W_0(ij)$ due to the dipole-dipole interactions between spin $\frac{1}{2}$ nuclei are computed (2) as

$$W_k(ij) = K_k \frac{\gamma_i^2\gamma_j^2\hbar^2}{r_{ij}^6} \frac{\tau_c}{1 + \Delta_k^2\tau_c^2}, \quad k = 0, 1, 2, \tag{10a}$$

where $K_0 = 0.1, K_1 = 0.15, K_2 = 0.6$, and $\Delta_0 = (\omega_i - \omega_j)$, $\Delta_1 = \omega_i$ and $\Delta_2 = (\omega_i + \omega_j)$; ω_i and γ_i are the Larmor frequency and magnetogyro ratio, respectively, for spin i; τ_c is the rotational correlation time and $\hbar$ is $\frac{1}{2}\pi$ times Planck's constant. A homonuclear spin system yields a further simplification

$$W_k(ij) = (1/r_{ij}^6) \cdot w_k, \tag{10b}$$

with

$$w_k = (\gamma^4\hbar^2\tau_c)K_k/(1 + (k\omega\tau_c)^2), \quad k = 0, 1, 2. \tag{11}$$

The w_k may be regarded as transition probabilities for a pair of spins separated by a unit distance. With Eqs. 10 and 11, the set of simultaneous equations (6) can be reexpressed, in a form more suitable for computation as

$$A \cdot F = C \tag{12a}$$

or

$$F = A^{-1} \cdot C \tag{12b}$$

where A is a square matrix of dimension $N - n_s$ (with the indices representing all the spins except the saturated spins), where n_s is the number of saturated spins. The diag-

onal elements are given by

$$A_{ii} = \sum_{m}{}' r_{im}^{-6} + \rho_i^{**}. \tag{13}$$

The prime on the summation indicates $m \neq i$. The off-diagonal elements are

$$A_{ij} = \eta r_{ij}^{-6}, \quad j \neq s. \tag{14}$$

In these equations

$$\rho_i^{**} = \rho_i^{*}/(w_2 + 2w_1 + w_0), \tag{15}$$

and

$$\eta = (w_2 - w_0)/(w_2 + 2w_1 + w_0). \tag{16}$$

F and C are column matrices with elements

$$F_i = f_i(S),$$

$$C_i = \eta \sum_{s} r_{is}^{-6}. \tag{17}$$

The parameter η represents the NOE observed when the spin system consists of only an isolated pair of spins. Using Eq. 11, we can express η in terms of τ_c and ω as (34, 11)

$$\eta = \frac{5 + \omega^2\tau_c^2 - 4\omega^4\tau_c^4}{10 + 23\omega^2\tau_c^2 + 4\omega^4\tau_c^4}. \tag{18}$$

η is independent of r_{ij}, giving the well-known result that the NOE for an isolated pair of spins relaxing solely through dipolar interaction does not give information about the internuclear distance. Since η is completely symmetric both in ω and τ_c, this implies that the NOE, $f_i(S)$, for a general case is symmetric with respect to changes in these two parameters, provided that ρ_i^{**} in Eq. 13 is negligible. This result has important consequences for proteins and polypeptides (*vide infra*). For small molecules satisfying the extreme narrowing approximation ($\omega^2\tau_c^2 \ll 1$), $\eta = 0.5$, and for large proteins where $\omega\tau_c \gg 1$, $\eta = -1$.

In systems where for a given spin i the indirect contribution to the NOE $f_i(S)_{\text{ind}}$, is negligible, the analysis of NOE data is considerably simplified. Let $f_i(m)$ and $f_i(n)$, respectively, be the NOE's observed for spin i when two neighboring spins m and n are saturated. If indirect contributions (Eq. 9) are negligible, then we obtain from Eqs. 7, 8, 2, and 10:

$$f_i(m)/f_i(n) = \sigma_{im}/\sigma_{in} = (r_{in}/r_{im})^6 \tag{19a}$$

or

$$r_{in}/r_{im} = (f_i(m)/f_i(n))^{1/6}. \tag{19b}$$

Thus, Eq. 19*b* can be used to extract information about the molecular structure in terms of ratios of distances. However, it must be borne in mind that the assumption

of negligible contribution of $f_i(S)_{ind}$ in Eq. 7 to the total NOE should be rigorously established before such a simplified analysis with Eq. 19*b* is undertaken. For peptides and proteins with correlation times $\tau_c \geq 10^{-9}$ s the indirect contributions could be important. For simple spin systems, more exact expressions including the indirect contributions can be derived (2) for the ratio of distance.

Effect of Anisotropic Molecular Rotation

In the previous section we have shown that the assumption of isotropic rotational motion of the molecule introduces considerable simplification in the analysis of the NOE experiment since Eq. 6 can be simplified to Eq. 12 using the factorization indicated in Eq. 10*b*. For spin systems with negligible ρ_i^{**}, the NOE's, $f_i(S)$, are all functions of a single parameter η and of the various distances. In the case of anisotropic rotational diffusion of the molecule, each internuclear vector will have a different effective rotational correlation time depending upon its orientation with respect to the principal axes of the diffusion tensor of the molecule. The general problem of dipolar relaxation in a molecule undergoing anisotropic rotational diffusion has been considered in the literature (35–37). Here we shall confine ourselves to a discussion of the anisotropic rotational motion of symmetric top molecules since valinomycin-K^+ belongs to this class as a result of its threefold symmetry. In this case, the transition probabilities $W_2(ij)$, $W_1(ij)$, and $W_0(ij)$ for a pair of nuclei i and j (homonuclear) whose internuclear vector $\mathbf{r}_{ij}$ makes an angle θ_{ij} with the symmetry axis of the molecule are given by (35)

$$W_k(ij) = K_k \frac{\gamma^4\hbar^2}{r_{ij}^6} \times \left\{ \frac{P_{ij}\tau_P}{1 + k^2\omega^2\tau_P^2} + \frac{Q_{ij}\tau_Q}{1 + k^2\omega^2\tau_Q^2} + \frac{R_{ij}\tau_R}{1 + k^2\omega^2\tau_R^2} \right\} \text{with } k = 0, 1, 2, \qquad (20)$$

where $K_0 = 0.1$, $K_1 = 0.15$, and $K_2 = 0.6$ and

$$P_{ij} = \tfrac{1}{4}(3\cos^2\theta_{ij} - 1)^2,$$
$$Q_{ij} = 3\cos^2\theta_{ij}\sin^2\theta_{ij},$$
$$R_{ij} = \tfrac{3}{4}\sin^4\theta_{ij},$$
$$1/\tau_P = 6D_\perp,$$
$$1/\tau_Q = D_\parallel + 5D_\perp, \qquad (21)$$

and

$$1/\tau_R = 4D_\parallel + 2D_\perp; \qquad (22)$$

$D_\parallel$ is the diffusion constant for rotation about the symmetry axis, and $D_\perp$ is the diffusion constant for rotation about an axis perpendicular to the symmetry axis. The NOE's can be computed from Eqs. 20, 2–4, and 6. Fig. 1 shows the effect of anisotropic rotation of a symmetric top molecule on the NOE for an isolated pair of spins relaxing through mutual dipolar interaction. It is seen that the NOE is severely

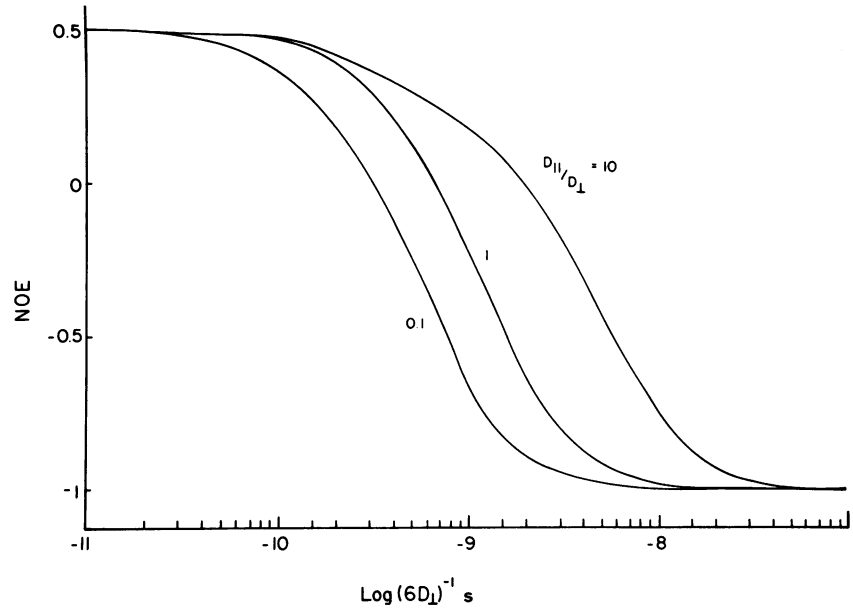


FIGURE 1 Effect of anisotropic rotational diffusion on the homonuclear NOE enhancement for an isolated pair of spins in a symmetric top molecule. $D_{\parallel}$ and $D_{\perp}$ are the two diffusion constants. The internuclear vector makes an angle $\pi/2$ with the symmetry axis. Operating frequency 270 MHz.

affected whenever rotational motion of the molecule is substantially anisotropic. If $D_{\parallel} = D_{\perp} = D$, we have isotropic rotational diffusion, and Eq. 20 reduces to Eq. 10*b* with $\tau_c = 1/6D$. For any proposed conformation the distances r_{ij} are known and the parameters θ_{ij} can be calculated since for symmetric top molecules the principal axis system of the diffusion tensor coincides with that of the moment of inertia tensor. Thus, the only two unknown parameters are $D_{\parallel}$ and $D_{\perp}$. By performing NOE experiments on a symmetric top molecule of known geometry, one can estimate $D_{\parallel}$ and $D_{\perp}$ through least squares fitting of experimental NOE data. In the extreme narrowing limit Eq. 20 reduces to

$$W_k(ij) = K_k \frac{\gamma^4 \hbar^2}{r_{ij}^6} \times \left\{ \frac{(3\cos^2\theta_{ij} - 1)^2}{24 D_{\perp}} + \frac{3\cos^2\theta_{ij}\sin^2\theta_{ij}}{D_{\parallel} + 5D_{\perp}} + \frac{3\sin^4\theta_{ij}}{4(4D_{\parallel} + 2D_{\perp})} \right\}. \quad (23)$$

The quantity in the brackets { } denotes the effective correlation time for the internuclear vector $\mathbf{r}_{ij}$ in the limit of extreme narrowing. The importance of anisotropic reorientation in the case of valinomycin-K^+ may be judged approximately by comparing the values of the three principal components of the moment of inertia tensor. These values are computed (for the C-I structure in ref. 16) to be $I_x = I_y =$ 26,906 $\times 10^{-40}$ $g \cdot cm^2$, and $I_z =$ 35,358 $\times 10^{-40}$ $g \cdot cm^2$, with the *z*-axis oriented

along the symmetry axis. We will also assume that the angular velocity correlation times about these three axes (37) do not differ significantly. Since the I_x and I_y differ from I_z by only about 25%, it is reasonable to assume that the rotational motion of valinomycin-K^+ may be described to a good approximation as isotropic rotational diffusion, and we can use the set of Eq. 10 to compute the transition probabilities. This view is further substantiated by the fact that the relaxation times for the various backbone $^{13}C^{\alpha}$ spins of valinomycin-K^+ in CD_3OD are all equal within experimental error (38). However, it is clear that in general, for peptides and proteins whose molecular shape can deviate substantially from spherical symmetry, one should also take into consideration the general problem of anisotropic rotational motion in describing the nuclear spin relaxation times or NOE's.

Conformational Averaging

Quite often the peptide under investigation does not have a single preferred conformation but exists under dynamic equilibrium between different conformations. The problem of computing the NOE enhancements in the extreme narrowing limit for a molecule undergoing conformational averaging has been studied by Schirmer et al. (39). In this section we will extend their analysis to the general case appropriate to larger molecules (e.g. peptides). Let the various possible conformational states of the molecule be denoted as α, β, δ, ... with n_α, n_β, ... as the corresponding fractional populations ($\sum_\gamma n_\gamma = 1$). Treating the conformational changes as chemical exchange between the conformational states, one can rewrite Eq. 1 including exchange terms in the manner of McConnell (40). Furthermore, it will be assumed that the exchange is fast on chemical shift time scale so that only one resultant spectrum is observed. We will examine the problem under two limiting cases treated by Schirmer et al. (39):

(*a*) The exchange rate is slow compared to relaxation rates. In this limit, one obtains

$$f_i(S) = \sum_\gamma n_\gamma f_i(S, \gamma) \tag{24}$$

where $f_i(S)$ is the resultant NOE enhancement, while $f_i(S, \gamma)$, the NOE enhancement in the γ^{th} conformation, can be computed by solving a set of equations analogous to Eq. 6. The observed NOE is simply the weighted average NOE of all the conformations.

(*b*) The exchange rate is fast compared to the relaxation rates. In this limit, the set of simultaneous Eq. 6 must be replaced by

$$\sum_j^n <\sigma_{ij}> f_j(S) = \sum_s <\sigma_{is}>, \tag{25}$$

where $f_j(S)$ is the resultant NOE enhancement and

$$<\sigma_{ij}> = \sum_\gamma n_\gamma \sigma_{ij}^\gamma. \tag{26}$$

σ_{ij}^γ denotes the relaxation rate in the γ^{th} conformation. This parameter can be computed using equations analogous to equations 2–4.

If the experimental NOE data cannot be explained by any single conformation, it may be possible to interpret the data in terms of a number of conformations by employing either Eq. 24 or Eq. 25, depending upon the rate of the conformational transitions. Fortunately, within the limits of experimental precision, the valinomycin-K^+ NOE data can be fit in terms of a single conformation (*vide infra*), and the uncertainties inherent in conformational averaging can be avoided. However, for a more typical case, e.g., uncomplexed valinomycin in DMSO-d_6, one should consider the possibility of conformational averaging.

Nuclear Spin Diffusion and Transient NOE (TNOE) Experiments

NUCLEAR SPIN DIFFUSION The NOE experiment is most useful when it discriminates between spins on the basis of their distances from the saturated spin. As may be seen from Eqs. 2–6, the enhancement depends on the transition probabilities $W_2(ij)$, $W_1(ij)$, and $W_0(ij)$ given in Eq. 10*a*. For homonuclear spin systems (Eq. 10*b*), $W_0(ij)$ is independent of the resonance frequency (and field) while $W_2(ij)$ and $W_1(ij)$ exhibit a strong frequency dependence. $W_2(ij)$ and $W_1(ij)$ do not con-

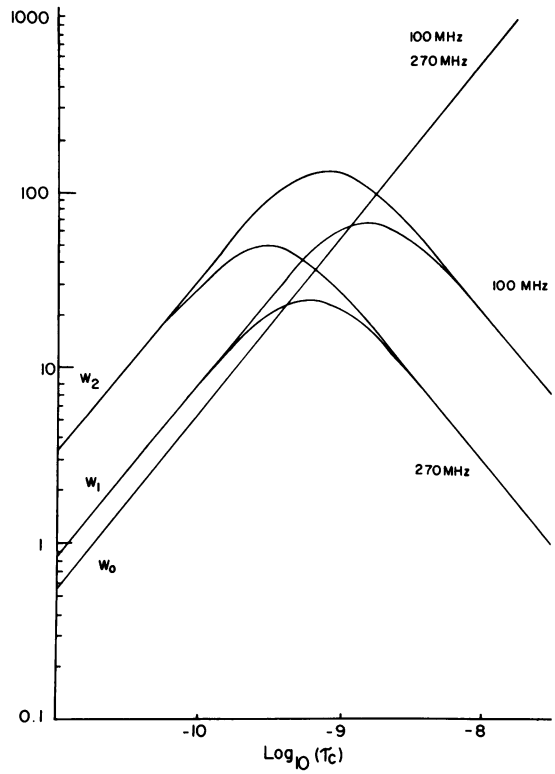


FIGURE 2 Variation of the dipolar transition probabilities for a pair of protons, as a function of the isotropic rotational correlation time τ_c. The protons are separated by 1 Å. The transition probabilities are computed at two different Larmor frequencies, 100 and 270 MHz.

serve the nuclear Zeeman energy and the nuclear spin transitions they induce are accompanied by exchange of energy with the lattice. On the other hand, $W_0(ij)$ conserves the Zeeman energy (for homonuclear spin systems) since it induces flip-flop transitions among the spins. Fig. 2 shows the variation of W_2, W_1, and W_0 as a function of τ_c at two different external magnetic fields. Whenever W_0 becomes the dominating process (for long correlation times and high external magnetic fields), efficient contact between the nuclear spins is established. Because of the strong coupling of nuclear spins with one another, any perturbation of one nuclear spin is quickly transferred to the rest of spins in the molecule. This phenomenon is referred to in literature as "nuclear spin diffusion." A lucid discussion of this phenomenon may be found in Chapters V and IX of Abragam (1). It becomes a dominating process for large correlation times of molecular motion (as in the case of solids) and at high external magnetic fields. Proteins and other large biomolecules generally exhibit a high degree of spin diffusion, which complicates the analysis of their NOE's and spin lattice relaxation times (3–7). This problem originates from both of the causes noted above (7)—long rotational correlation times ($\tau_c \geq 10^{-8}$ s), which endow proteins with many of the properties of solids, and high magnetic fields, which are employed to unravel the complex spectra generally exhibited by these macromolecules.

The effect of long correlation times or large external magnetic fields on the NOE enhancements of a homonuclear spin system (undergoing an isotropic molecular rotation) can be calculated with Eqs. 12–15. Let us consider the particular situation which results when ρ_i^{**} in Eq. 13 is negligible for all i. In this limit the only mechanism of relaxation for the nuclear spins is their mutual dipole-dipole interaction. The NOE's, $f_i(S)$, depend upon the relative magnitudes of various distances, r_{ij}, as well as on the parameter η. The dependence of η (the NOE for an isolated pair of spins) on the Larmor frequency ω and the correlation time τ_c is given in Eq. 18. When $\omega\tau_c \gg 1$, the parameter η has a value of -1 and the various NOE enhancements, $f_i(S)$, have a simple and unique solution: since the matrix elements A_{ij} in Eq. 12 satisfy the relation

$$\sum_j A_{ij} = -C_i, \tag{27}$$

it immediately follows that

$$f_i(S) = -1 \quad \text{for all } i \text{ and } s. \tag{28}$$

This result is a simple manifestation of the phenomenon of nuclear spin diffusion caused by the flip-flop term of the dipolar Hamiltonian for like spins, and in this limit (i.e., $\eta = -1$) the NOE experiment does not discriminate between spins on the basis of proximity to the saturation site or on the basis of molecular dynamics. It may, however, be possible to design transient NOE experiments sensitive to internuclear distances even in the spin diffusion limit (*vide infra*).

Whenever ρ_i^{**} is not negligible and whenever η deviates from -1, Eq. 27 is not realized and the NOE experiment may show discriminatory effects.

So far, in the above calculations and in deriving Eq. 27, we have assumed isotropic reorientation of the molecule, so that all internuclear vectors have a single correlation time. When there is a substantial anisotropic contribution to the dipolar relaxation rates, the conclusions drawn above for nuclear spin diffusion need to be re-examined since the propagation of nuclear spin diffusion along the various dipole-dipole vectors in a protein will also be anisotropic. In favorable cases it may be possible to exploit this aspect to gain structural information or to make resonance assignments.

TRANSIENT NOE (TNOE) EXPERIMENT The inherent limitations of the steady state NOE technique when applied to macromolecular systems have been considered in the previous section. For long rotational correlation times ($\gtrsim 10^{-8}$ s) typical of proteins, the steady-state NOE experiment is less sensitive in discriminating protons according to their geometrical arrangement in the molecule. This effect arises because, under steady state, the saturation of the irradiated proton is spread to the remaining protons in the protein through the process of nuclear spin diffusion. This problem could be overcome to some extent through transient experiments involving selective excitation of one of the spins and observation of the remaining spins. These experiments may be regarded as a generalization to multispin systems of the classic experiments performed by Solomon and Bloembergen (41). The process of nuclear diffusion (brought in by the flip-flop term of the dipolar Hamiltonian [1]) tends to establish a common spin temperature among the various protons by spreading the perturbation. If observations are made before all the protons acquire a common spin temperature, then discriminatory effects can be observed in the various proton resonances and useful structural information is obtained. The selective excitation or perturbation can be applied in several ways: (*a*) selective inversion of the magnetization of a spin; (*b*) selective progressive saturation of a chosen spin (i.e., switching on the decoupler for increasing periods of time, starting with very small periods, etc. To demonstrate the discriminatory effects obtained through the TNOE method, we have performed calculations on a three-proton system AMX. An operating frequency of 270 MHz and a rotational correlation time of 8×10^{-8} s (typical of proteins of the size of alkaline phosphatase [3, 4]) have been assumed. The experiment consists of selectively inverting the magnetization of proton X and monitoring intensity changes of all the three proton resonances. For short times (shown in Fig. 3) the M spin shows much larger intensity changes than A, thus providing discriminatory information. For intermediate times the magnetizations of all the spins approach a common value (this corresponds to the establishment of a common spin temperature) and they all relax together towards their equilibrium magnetizations through W_1, W_2 and ρ^* processes until the spin temperature equals the lattice temperature.[1] In favorable cases, a simple analysis of these experiments in terms of cross-relaxation terms, σ_{ij}

[1]In this case, the nuclear spins relax together with an average rate equal to the sum of average values of W_1, W_2, and ρ^* terms. In those instances where this average rate is much too small to be convenient for an experiment, it may be possible to enhance this rate by the addition of trace amounts of paramagnetic impurities since these contribute to the ρ^* terms.

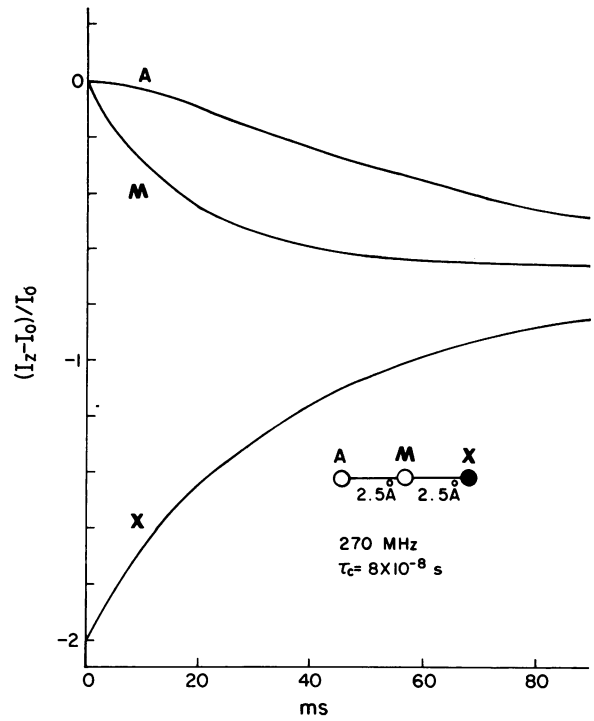


FIGURE 3 Transient NOE experiment involving selective excitation of spin X in a three-proton system AMX. The spins are 2.5 Å apart in a straight line. The experiment consists of selective inversion of the magnetization of spin X and monitoring the changes in the intensities of the magnetizations of all the spins as a function of time. The correlation time, 8×10^{-8} s, used in the calculations is typical of proteins of mol wt $\simeq$ 80,000. ^{1}H frequency 270 MHz.

(Eq. 2), is possible through the measurement of initial relaxation rates (33) for the various spins. The TNOE experiments can potentially be useful in extending the structural determination by NMR to proteins of size larger than those amenable to the steady-state NOE technique.

METHODS

Valinomycin-K^+ Complex

Valinomycin (59.4 mg) (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 2 ml of a solution of KBr in CH_3OH (3.18 mg/ml). KBr was obtained from Ventron Corporation, Beverley, Mass. Solvent was removed with a rotary vacuum evaporator ($3\frac{1}{2}$ h at 40°C). The dry residue was dissolved in 0.8 ml of $CDCl_3$ and centrifuged for 10 min, and about 0.6 ml of the clear solution was transferred to a 5 mm outside diameter NMR sample tube. The solution was degassed by several freeze-pump-thaw cycles and finally sealed under vacuum. The formation of the complex was confirmed by comparing the proton chemical shifts of this sample with that of a similar amount of uncomplexed valinomycin dissolved in $CDCl_3$. The direction and magnitude of complexation shifts for the NH, $C^{\alpha}H$ and $C^{\beta}H$ resonances agreed with data reported in the literature (42). In addition to these, the observed vicinal coupling constants ($J^{\text{L-Val}}_{\text{NH}-\text{C}^{\alpha}\text{H}}$ = 4.6

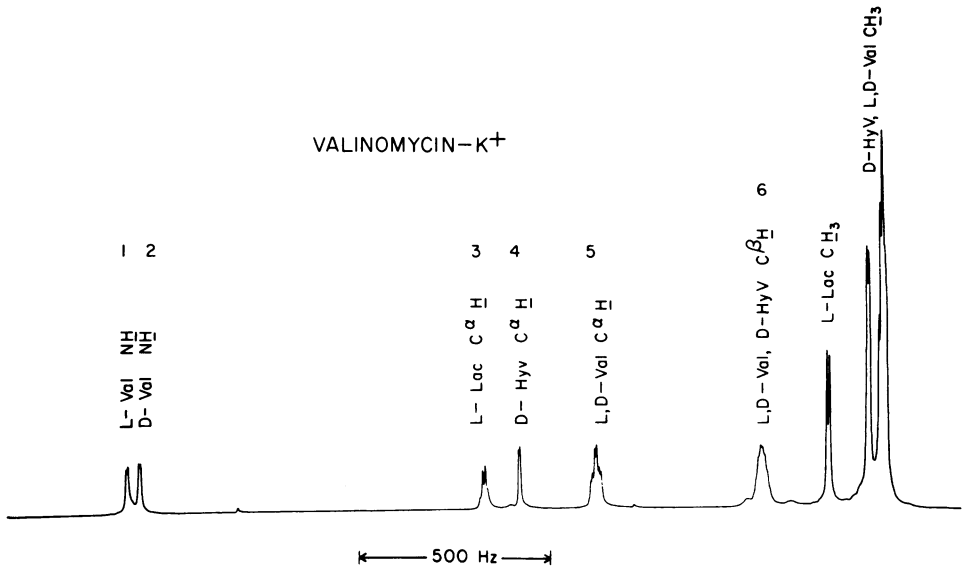


FIGURE 4 270 MHz ^{1}H NMR spectrum of valinomycin-KBr in $CDCl_3$. The different proton resonances are numbered from the left.

Hz; $J^{\text{D-Val}}_{\text{NH}-\text{C}^\alpha\text{H}}$ = 4.7 Hz; $J^{\text{L-Val}}_{\text{C}^\alpha\text{H}-\text{C}^\beta\text{H}}$ = 11.4 Hz; $J^{\text{D-Val}}_{\text{C}^\alpha\text{H}-\text{C}^\beta\text{H}}$ = 11.1 Hz; $J^{\text{D-Hyv}}_{\text{C}^\alpha\text{H}-\text{C}^\beta\text{H}}$ = 3.5 Hz)[2] are in reasonable agreement with the reported values for valinomycin-K^+ (43).

NMR Measurements

All the NOE experiments were performed in the pulsed Fourier transform mode on a 270 MHz NMR spectrometer. The NMR signal of interest was selectively saturated for a period of 6.2 s (this time was longer than 5 T_1, where T_1 is the longest of all the proton spin-lattice relaxation times). At the end of the saturation, the NMR spectrum was obtained by applying an observation pulse ($\pi/2$) and Fourier-transforming the resulting free induction decay (FID). The single resonance spectrum is obtained by shifting the decoupler frequency away from the spectral region. In the actual experiment about 32 FID's were accumulated for each experiment. A band width of 4,000 Hz with 8 K data points was used. A 2-Hz exponential line broadening was used in Fourier transforming the FID's. Finally the NOE's were obtained by subtracting the single-resonance Fourier transform-NMR spectrum from that of the double resonance.

RESULTS AND DISCUSSION

A typical 270 MHz proton NMR spectrum of valinomycin-K^+ complex is shown in Fig. 4. Fig. 5 shows the NOE results obtained for valinomycin in two different conformations: a typical NOE experiment on valinomycin-K^+ in $CDCl_3$ (performed in the present investigation) is shown in Fig. 5 A; the NOE experiment on uncomplexed valinomycin in DMSO-d_6 performed in a previous investigation (11) is shown in Fig.

[2]These are the observed values and have not been corrected for electronegativity effects.

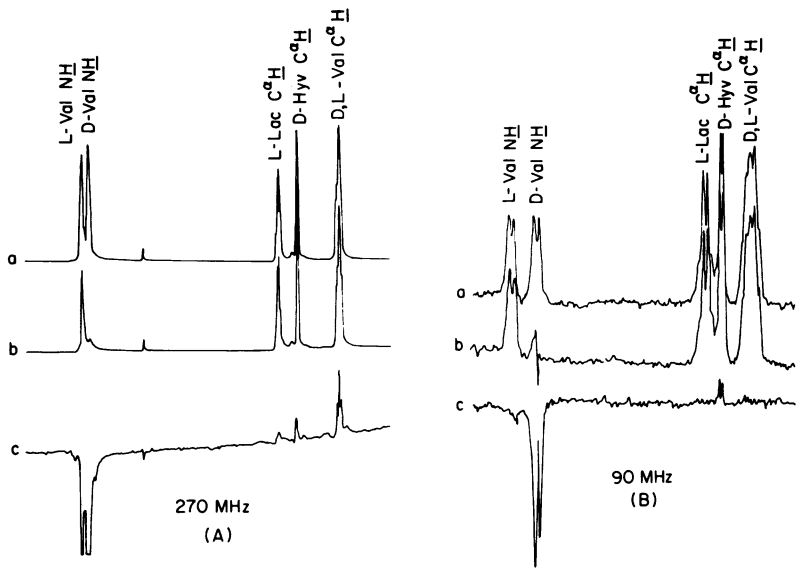


FIGURE 5 Intramolecular NOE measurements involving the saturation of D-val NH for valinomycin in two different conformations. (A) Valinomycin-K^+ in $CDCl_3$. ^1H NMR frequency 270 MHz. *a* is the control spectrum (single resonance), *b* is the double-resonance spectrum, and *c* is the difference (*b* – *a*) amplified eight times. (B) Uncomplexed valinomycin in $(CD_3)_2SO$. ^1H NMR frequency 90 MHz. *a* and *b* are the single- and double-resonance spectra, respectively, and *c* is the difference (*b* – *a*) amplified two times.

5 B. In both cases the NH resonance of D-Val is saturated. At 270 MHz the potassium complex exhibits positive NOE's, the largest NOE being exhibited by the multiplet consisting of L- and D-Val $C^{\alpha}H$ resonances with lesser enhancements of the L-Lac and D-Hyv $C^{\alpha}H$ resonances. In sharp contrast, uncomplexed valinomycin in DMSO-d_6 at 90 MHz yields no measurable NOE for the $C^{\alpha}H$ resonances of L- and D-Val, but shows a relatively large positive NOE for the $C^{\alpha}H$ resonance of D-Hyv residue. Significantly, this NOE becomes negative at 250 MHz (11). These results establish, unequivocally, the sensitivity of the NOE experiment to the conformation of a peptide in solution and to the resonance frequency. The experimental NOE data for valinomysin-K^+ is shown in Table I. It includes the experiments involving the observation and saturation of all the backbone protons (NH and $C^{\alpha}H$) and the $C^{\beta}H$ protons of the side chain. Since these experiments produced sufficient data for conformational analysis (*vide infra*), experiments involving the methyl groups were not considered in the analysis. The experimental NOE enhancements were analyzed by comparing them with the NOE values calculated for each of the proposed conformations of valinomycin. This objective was accomplished in two steps: (*a*) generation of the structure of valinomycin using the proposed torsional angles and (*b*) optimization of τ_c for each model to yield the best agreement between the experimental and the calculated NOE's.

TABLE I

EXPERIMENTAL NOE ENHANCEMENTS* AT 270 MHz FOR VALINOMYCIN-K^+ IN $CDCl_3$ (30°C)

Saturate‡§	Observe ‡					
	1	2	3	4	5	6
1	—	‖	0.02		0.05	0.03
2	‖	—	0.01	0.02	0.04	0.03
3			—	¶	¶	
4	0.02		¶	—	¶	0.04
5	0.03	0.04			—	0.02
6	0.05	0.05	0.05	0.13		—

*This table shows the NOE values (±0.01) that have been included in the analysis. These are the average of at least four or more separate measurements.

‡The numbers 1, 2 . . . 6 etc. refer to the NMR peaks in Fig. 4.

§Saturated peak indicated by a horizontal bar.

‖ Peak too close to the irradiated one.

¶ Measurements made difficult by glitches and other artifacts.

Generation of the Valinomycin Structure

Computer generation of the structure of valinomycin presents certain problems unique to cyclic peptides. Our initial attempts consisted of using the torsional angles proposed for each conformation in literature. These literature values along with the notation for each model have been summarized in Table II of ref. 11. We will follow the same notation in this paper also as far as possible, except for a few conformations recently updated by Bystrov et al. (43). The entire structure was generated by straightforward procedures, starting at a particular atom and using the appropriate translations and rotations to generate the remaining atoms. Standard bond lengths and bond angles were used.[3] We found that practically none of the structures produced perfect closure. This was not unexpected since the reported ϕ angles were obtained either through the use of the Karplus curve (44, 45) or from potential energy calculations and molecular models, while the ψ torsional angles were computed from the potential energy calculations and from molecular models. These values are expected to have inherent uncertainties of the order of ±5° or more, thus leading to a failure of ring closure for valinomycin. Our next attempt, and a successful one, consisted of adapting to valinomycin the procedure of Go and Scheraga (46) for generating cyclic structures with automatic ring closure. Their procedure was used to refine the torsion angles for each of the proposed structures. Due to the threefold symmetry of the molecule, the entire conformation can be specified by means of the eight torsion angles ϕ_1, ψ_1, ϕ_2, ψ_2, ϕ_3, ψ_3, and ϕ_4, ψ_4 defining the backbone conformation of the residues L-Val, D-Hyv, D-Val, and L-Lac, respectively. Since the angles ϕ_1 and ϕ_3 reported in the liter-

[3]The bond lengths in Ångstroms are: N—C^α = 1.466; C^α—C′ = 1.518, C′—N = 1.329; C—H = 1.09; C′=O = 1.244; O—C^α = 1.458; C^α—C′ (Ester) = 1.519; C′—O = 1.344, C′=O (Ester) = 1.206; N—H = 1; C^α—C^β = 1.525; C^β—C^γ = 1.525. The bond angles in degrees are: C^α—C′=O = 117.8; C^α—C′—N = 120.7; C′—N—C^α = 119.5; N—C^α—C′ = 107.6; C^α—C′=O (ester) = 125.2; C^α—C′—O = 111.5; C′—O—C^α = 117.2; O—C^α—C′ = 111.6.

TABLE II

OPTIMIZED TORSION ANGLES FOR VARIOUS PROPOSED CONFORMATIONS OF VALINOMYCIN IN SOLUTION

Designation*			L-Val		D-Hyv		D-Val		L-Lac	
1	2	3	$\phi 1$	$\psi 1$	$\phi 2$	$\psi 2$	$\phi 3$	$\psi 3$	$\phi 4$	$\psi 4$
	A_1	I‡	30	57.938	98.23	−23.08	−40	−55.608	−96.699	23.805
		A_1§	25	70.651	100.54	−20.052	−40	−69.32	−99.6	30.438
A		C-I‡	−70	107.76	68.079	0.82042	70	−107.76	−68.079	−0.82042
	A_2	K‖	−58.6	131.33	80.481	4.875	57.8	−131.16	−72.236	−21.367
		KU¶	−68	120.08	94.074	4.5618	68	−120.07	−94.075	−4.5611
		AU¶	−80	91.542	120.43	−13.695	90	−93.448	−123.21	12.294
		BU¶	−85	106.76	123.43	−15.92	110	111.67	−100.35	110.7
B	B_1	II-1‡	30	90.405	98.158	−59.251	140	116.24	−83.957	87.462
		B§	50	62.534	97.458	−39.999	120	86.383	−58.341	118.86
	C_1	III-1‡	−145	107.41	88.041	−72.758	145	−103.15	−92.417	75.253
C	C_2	C-II‡	−160	161.94	111.93	−51.4	160	−161.94	−111.93	51.399
	C_3	III-2‡	−90	119.84	73.786	−150.9	90	−126.76	−64.418	148.32

Torsion angles (ϕ, ψ) are designated in accordance with the convention established by the IUPAC-IUB Commission on Biochemical Nomenclature (J. C. Kendrew, W. Klyne, S. Lipson, T. Miyazawa, G. Nemethy, D. C. Phillips, G. N. Ramachandran, and H. A. Scheraga. 1970. *J. Mol. Biol.* **52**:1.

*The various torsional angles are obtained after optimizing the literature values (listed in Table II of ref. 11 and Table 7 in ref. 43) to produce a perfect closure using the listed bond angles and bond lengths in footnote 2. The optimization was done in a least squares sense with respect to the literature values (see text). The correspondence between the optimized torsion angles and literature values is obtained by comparing the angles in this table with those listed in refs. 11 and 43. Columns 1 and 2 under Designation give the broad general classification and subclassification, respectively. Column 3 gives the designation used in the literature (exception KU, AU, and BU).

‡Ref. 16.

§Ref. 17.

‖Ref. 22.

¶Ref. 43. These three conformations (KU, AU, and BU) are listed as K, A, and B in this reference, but we have added the letter U to indicate that these models are refined or updated models.

ature were obtained in most cases through the measurement of vicinal $J_{NH-C^{\alpha}H}$ coupling constants, these two values were taken as more reliable than others and were held fixed. The remaining angles ψ_1, ϕ_2, ψ_2, ψ_3, and ϕ_4, ψ_4 were optimized in a least squares sense with respect to the proposed torsional angles so that the identity of each of the proposed conformations was preserved. Table II gives the various backbone torsional angles obtained in this manner for most of the models listed in Table II of ref. 11 (we have excluded the B_2 and D structures). These angles give exact ring closure for valinomycin. We have also included in Table II the refined torsional angles (models KU, AU, and BU) for valinomycin recently updated by Bystrov et al. (Table 7 of ref. 43) through the use of homo and heteronuclear vicinal coupling constants. In addition to the backbone torsion angles, we also need to specify the torsion angles χ_1, χ_2, and χ_3 for the orientation of the side chains of the three residues L-Val, D-Hyv, and D-Val, respectively. For the A (43, 30) and C structures we have assumed that $\chi_1 = \chi_3 = 180°$ and $\chi_2 = 60°$. For the B structures we have assumed that $\chi_1 = 180°$ and $\chi_2 = \chi_3 = 60°$, as suggested by the recent studies (43). The di-

polar interactions between the protons within a methyl group were calculated by neglecting internal rotation and assuming that these protons have the same overall rotational correlation time, τ_c, as the rest of the molecule. For all other dipolar interactions, the methyl group protons were assumed to be located at the center of an equilateral triangle formed by these protons. This is a reasonable approximation to the modulation of internuclear vectors due to the fast interal rotation of the methyl groups, especially for all protons not directly adjacent to the methyl group (48).

NOE Calculations and Results

For each of the conformations in Table II, the NOE enhancements were calculated as a function of correlation time τ_c using Eq. 12. The parameter ρ_i^{**} was set to zero for all the protons except the NH protons of L-Val and D-Val since these protons can experience significant dipolar interaction with the ^{14}N nucleus. For these two protons ρ_i^{**} is given by Eq. 15, where

$$\rho_i^* = \rho_{NH}^* = \frac{8}{3}\frac{\gamma_N^2\gamma_H^2\hbar^2\tau_c}{r_{NH}^6} \cdot\left\{\frac{0\cdot1}{1+(\omega_N-\omega_H)^2\tau_c^2}+\frac{0\cdot3}{1+\omega_H^2\tau_c^2}+\frac{0\cdot6}{1+(\omega_N+\omega_H)^2\tau_c^2}\right\} \quad (29)$$

All the computations were performed on an IBM 370 computer (IBM Corp., White Plains, N.Y.) using SPEAK-EASY (48). For each model the correlation time, (τ_c), was optimized in a least squares sense using the Marquardt algorithm (49) to minimize the goodness of fit parameter χ^2 defined by (50)

$$\chi^2 = \frac{1}{(N_x - N_p)}\sum_{i=1}^{N_x}\left(\frac{f_{io}-f_{ic}}{\sigma_i}\right)^2, \quad (30)$$

where f_{io} and f_{ic} are the observed and calculated NOE values, σ_i the standard deviation, N_x the total number of NOE measurements included in the fit, and N_p the number of parameters used in the optimization. The results of the least squares analysis are shown in Table III, which lists the various models, the minimum values of χ^2, and the correlation times corresponding to the best fit. In addition we have also listed for each model Hamilton's (51, 52) agreement factor R, defined by

$$R = \left[\frac{\sum_{i=1}^{N_x}\left(\frac{f_{io}-f_{ic}}{\sigma_i}\right)^2}{\sum_{i=1}^{N_x}\left(\frac{f_{io}}{\sigma_i}\right)^2}\right]^{1/2} \quad (31)$$

The use of the agreement factor as a basis of statistical hypothesis testing in X-ray crystallographic studies has been discussed by Hamilton (51, 52). Subsequently Wilcott et al. (53) and Agresti et al. (50) applied this agreement factor criterion to NMR studies of metal complexes. In this investigation we will use it as a basis for selecting models in agreement with the experimental NOE data and rejecting others at a given

TABLE III

ANALYSIS OF THE INTRAMOLECULAR NOE ENHANCEMENTS OF VALINOMYCIN-K^+ IN $CDCl_3$

	A						B			C		
	A_1		A_2				B_1			C_1	C_2	C_3
	I	A_1	C-I**	K	KU	AU	BU	II-1	B	III-1	C-II	III-2
χ^{2*}	9.27	7.8	1.59 (4.42)	2.506	2.079	1.99	9.48	10.26	11.18	9.025	6.125	11.985
R‡	0.631	0.579	0.261 (0.436)	0.328	0.299	0.292	0.638	0.663	0.693	0.622	0.513	0.717
R-ratio§	2.418	2.218	—	1.257	1.146	1.119	2.444	2.54	2.655	2.383	1.966	2.747
Confidence level ‖	99.5	99.5	—				99.5	99.5	99.5	99.5	97.5	99.5
τ_c¶	2.86	2.28	1.81 (3.1)	2.95	2.632	2.61	3.33	3.3	3.64	2.9	2.23	3.78

The 16 NOE enhancements in Table 1 were used. We have assumed a uniform standard deviation of σ_i = 0.01. The notation for the various models is same as in Table II.

*$\chi^2 = [\sum_i((f_{io} - f_{ic})/\sigma_i)^2]/(N_x - N_p)$; the values reported are the minimum values in the fit.

‡R is the agreement factor defined by $R = [\sum_i((f_{io} - f_{ic})/\sigma_i)^2/\sum_i(f_{io}/\sigma_i)^2]^{1/2}$. (Ref. 52.)

§R-factor ratio obtained by dividing each R value by the smallest R value (=0.261) corresponding to the C-I model.

‖ Indicates the confidence level for rejection.

¶ Correlation time in units of 10^{-10} s that corresponds to the best agreement between experiment and theory (i.e. minimum χ^2 value).

**Values in brackets are obtained by neglecting ^{14}N-^1H dipolar interaction for the NH protons of L-Val and D-Val.

level of confidence. An examination of Table III shows that the A_2 models (C-I, KU, K, and AU) have considerably smaller values of χ^2 and R compared to the rest of the models. The R factor values for the A_2 models range from 0.26 to 0.3, indicating that the experimental NOE values deviate from that of theoretical models on the average by 26–30%. We consider this as an indication that the torsional angles for these A_2 models need to be refined slightly to yield better agreement between experiment and theory. Nevertheless it is still possible to perform statistical hypothesis testing to see if each of the remaining models could be accepted or rejected with respect to the A_2 models in explaining the NOE data on valinomycin-K^+. This is done (52) as follows: Each model is in turn compared with the C-I model that yielded the lowest R factor (= 0.261). For each pair of models we formulate the null hypothesis: that the two models are equally valid, the differences in the fit being solely due to experimental error. To test this hypothesis, the R factor ratio is computed by dividing the R factor of the alternate model by 0.261. In fitting the experimental data (16 NOE's) only one parameter, the correlation time τ_c, was optimized for each model described by a set of 11 fixed parameters (4 ϕ values, 4 ψ values, and 3 χ values). Thus, the number of degrees of freedom equals 15. As a conservative estimate, the dimension of the hypothesis is taken as 12. Using the tables for the significance points of the R-factor ratio (51, 52), we can compute the confidence level with which the null hypothesis can be re-

jected. Table III gives the R-factor ratios and the confidence level for each of the models. The two A_1 models (I, A_1), the three B models (BU, II-1, B), and the C_1 and C_3 models could all be rejected with greater than 99.5% confidence. The C_2 model could be rejected with 97.5% confidence. Among the A_2 models, the conformations AU, KU, and K (the X-ray crystallographic structure [22] for valinomycin-K^+) cannot be rejected even at 0.5 significance level, and hence on a statistical basis they are as successful as the C-I model in explaining the NOE data. This procedure demonstrates the power of the NOE technique in picking models consistent with experimental data from among a set of alternate conformations. Use of the statistical hypothesis testing techniques permitted us to discriminate between various models on the basis of experimental data, in spite of the fairly large experimental errors associated with small NOE's. For the C-I conformation we have also included in brackets in Table III the parameters obtained by setting $\rho^*_{NH} = 0$ (Eq. 29) for the NH protons of L-Val and D-Val. It is seen that the inclusion of ρ^*_{NH} substantially improves the agreement between experiment and theory. Table IV compares the experimental NOE values with best fit values calculated for the C-I conformation with $\tau_c = 1.81 \times 10^{-10}$ s.

An interesting result of this analysis is that the model proposed by Bystrov et al. (43) for the uncomplexed valinomycin in nonpolar solvents (model AU in Table II) is also as successful as the models for the potassium complex (C-I, K, and KU) in explaining the NOE data. They both possess the A_2 "bracelet" type of structure with the amino acid carbonyls oriented towards the symmetry axis. In spite of the apparent similarity between the two structures, the torsion angles for the AU structure differ somewhat from that of valinomycin-K^+. This results from electrostatic repulsion among the

TABLE IV

COMPARISON OF EXPERIMENTAL ENHANCEMENTS (f_o) FOR VALINOMYCIN-K^+ IN $CDCl_3$ AND THEORETICAL NOE VALUES (f_c) COMPUTED FOR THE C-I MODEL (TABLE II) WITH $\tau_c = 1.81 \times 10^{-10}$ s

$a\{b\}$	f_o	f_c
14	0.02	0.01
15	0.03	0.03
16	0.05	0.06
25	0.04	0.03
26	0.05	0.05
31	0.02	0.01
32	0.01	0.01
36	0.05	0.07
42	0.02	0.01
46	0.13	0.12
51	0.05	0.03
52	0.04	0.03
61	0.03	0.01
62	0.03	0.01
64	0.04	0.04
65	0.02	0.01

a refers to the observed peak and {*b*} refers to the saturated peak in Fig. 4.

ester carbonyls in the absence of the cation that neutralizes their charges (43). Due to this repulsion, the ester carbonyls assume a more axial orientation. The success of both the models in explaining the NOE data indicates that, in spite of the difference in the torsion angles, the dipolar interactions have not changed substantially from one conformation to the other, at least as far as the 16 NOE measurements included in this analysis. This is reasonable since the two residues D-Hyv and L-Lac do not possess amide NH protons on the backbone and hence variations in ϕ_2 and ϕ_4 do not significantly change the dipolar interactions involving the $C^{\alpha}H$ protons of these two residues. In order to test whether the AU model is realized for uncomplexed valinomycin in nonpolar solvents one needs to perform NOE experiments on this system.

A determination of the backbone conformation of a peptide should include NOE experiments involving not only the backbone protons but also the side chain protons since there could be strong dipole-dipole interactions between these protons. Neglect of the side chain protons could lead to misleading conclusions in the interpretation of NOE experiments involving the backbone protons. Another experimental parameter extensively used in solution conformation studies is the vicinal coupling constant between two protons. This obeys a Karplus type of relation (44, 45) with respect to the dihedral angle. A combined use of the NOE and vicinal coupling constant data could remove some of the ambiguities associated with either technique. Such a study, involving the optimization of torsion angles and correlation times to obtain best fit with NOE's and vicinal coupling constants, is currently in progress in our laboratory.

Conclusions

A general formalism for the description of the intramolecular NOE experiment on a multispin system has been presented. This formalism is applicable to a wide variety of molecules (small organic molecules, peptides, and proteins). The assumption of isotropic molecular rotation without conformational averaging simplifies the calculations considerably.

Among the factors that can complicate the analysis of NOE experiments, we have considered (*a*) anisotropic molecular rotation, (*b*) conformational averaging, and (*c*) nuclear spin diffusion. In case *a* the theory of dipolar relaxation rates in the presence of anisotropic molecular rotation (35–37) should be incorporated into the NOE formalism. Using Woessner's formalism (35), we have shown that the NOE for an isolated pair of spins in a symmetric top molecule depends strongly on the degree of anisotropy of molecular motion. Such effects may be important for those peptides and proteins whose molecular shape deviates substantially from spherical symmetry. The formalism of Schirmer et al (39) to treat the effect of conformational averaging on the NOE has been extended to include molecules that do not satisfy the extreme narrowing limit of molecular motion. Nuclear spin diffusion limits the sensitivity with which the NOE experiment can distinguish between protons according to their geometrical arrangement in the molecule. It becomes a dominating process at large rotational correlation times (typical of proteins) and high external magnetic fields (i.e. $\omega\tau_c \gg 1$). To gain structural information in large proteins, transient NOE ex-

periments could be more useful than the steady-state NOE experiment. In the TNOE method one of the protons in the protein is selectively perturbed, and observations are made on the various proton resonances before they all acquire a common spin temperature. Such experiments provide discriminatory information even in the presence of a high degree of nuclear spin diffusion and hence may potentially be useful in extending NMR structural determinations to proteins beyond those accessible to the steady-state NOE techniques.

Our study of valinomycin-K^+ in $CDCl_3$ indicates that its conformation is consistent with the A_2 models (models C-I, K, KU, AU in Table II) proposed in the literature. An isotropic rotational diffusion model seems to provide an adequate description for the NOE's observed in this inophore-metal complex. Incorporation of $^{14}N-^1H$ dipole-dipole interactions for the NH protons of L-valine and D-valine improves the agreement between experimental and calculated NOE's. Using statistical hypotheses-testing criterion involving Hamilton's *R*-factor ratios, models other than A_2 have all been rejected as inconsistent with the experimental data. Our approach to the determination of peptide conformation consisted of a global analysis of all the NOE's rather than the use of isolated NOE experiments. For the C-I model (16) a correlation time, $\tau_c = 1.81 \times 10^{-10}$ s, gives the best fit between experimental and calculated NOE's. This value is consistent with 1.6×10^{-10} s reported by Komoroski (38) from the ^{13}C relaxation time studies of this complex in methanol.

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