Flexibility and Conformations of Guanosine Monophosphates by the Overhauser Effect

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Abstract: Distances of H_8 to the sugar protons were measured from proton–proton Overhauser effects and the relaxation rate of H_8. The results show that the guanosine phosphates are flexible in solution. Using a simple model to interpret the Overhauser enhancements in a flexible system, the guanosine phosphates are shown to be in syn conformations a large part of the time, confirming previous suggestions based on circular dichroism studies. Lowering the pD favors the syn conformations. The anti conformations are favored in aggregates, as is the case in crystals. The sum of the Overhauser enhancements is shown to be sensitive to small quantities of large aggregates which might pass unnoticed by other approaches.

1. Introduction

There have been many studies of nucleotide conformations. The conformations in crystals have been discovered by X-ray diffraction. The conformation of deoxyribonucleotide moieties in DNA (A and B forms) is also rather well determined by the same method.[2] However, in RNA (*e.g.*, tRNA) and perhaps even in DNA, the nucleotide conformation may not be uniform, the characteristic angles varying according to the nature of the neighboring bases and the local structure of the polymer.[3]

The structures of mono- and oligonucleotides in solution have been studied by nmr, using proton chemical shifts to investigate the relative positions of base and sugar, and the J couplings to derive possible sugar conformations.[4-8] A recent study based on nmr of rare earth complexes of nucleotides gives evidence of conformations similar to those observed in crystals.[9]

Other workers have used circular dichroism (CD) to investigate the structures of nucleic acid derivatives.[10-13] The weaker dichroism of the purine nucleotides, as compared to the pyrimidines, has been taken as evidence of greater mobility of the base with respect to the sugar in the former case.[13] For guanine derivatives it has been observed that the CD spectrum inverts upon protonation of the base; this was interpreted as a change from the anti to the syn conformation.[12]

Figure 1 is a representation of 5′-guanosine monophosphate (Guo-5′-P), showing some of the conformational features. The base is planar and will be considered rigid. It is linked to the sugar by the N_9–$C_{1'}$ glycosidic bond. The orientation of the purine plane is characterized by its angle ϕ_{CN} with the plane containing the N_9–$C_{1'}$ and $C_{1'}$–O bonds.[14a,b] Rotation around the glycosidic bond brings the base C_2 carbon away from (anti; $\phi_{CN} \approx -30°$) or toward (syn; $\phi_{CN} \approx +150°$) the ribose. The ribose is more precisely a β-D-ribofuranose; the base which substitutes on 1′ and the $C_{5'}$ carbon are on the same side of the sugar (above) while the 2′- and 3′-hydroxyl groups are below. To define the sugar geometry, two sets of parameters must be given. First, one must define the geometry of the ribose ring;[4b,8] for instance, we can have a 2′-endo situation in which $C_{1'}$, $C_{3'}$, $C_{4'}$, and O are coplanar, while the 2′ carbon is above this plane and $H_{2'}$ is nearly axial. Second, one must give the orientation of the 5′-CH_2OH group with reference to the trihedron formed by $C_{4'}$–$C_{5'}$, $C_{4'}$–O, $C_{4'}$–$C_{3'}$; here, three rotamers[8] are possible. It is generally considered that the two structure parameters just described may vary so that the sugar is correlatively flexible.

Lastly, phosphorylation introduces two new degrees of freedom. In the >P-O-$C_{n'}$ linkage (n = 2, 3, or 5), one must specify the orientations of the P–O bond and of the phosphate group as a whole relative to the tetrahedral bonds of $C_{n'}$.

It is clear that, depending on the various angles, the nucleotide can take very different conformations, from extended ones with the phosphate stretched away from the base, to compact ones with the phosphate, sugar, and base lumped together.

In the present work, we examine the structure of guanosine monophosphates by a magnetic relaxation method,[15,16a-e] the proton–proton Overhauser effect.[17]

(1) (a) Service de Biochemie; (b) Groupe de Biophysique.
(2) (a) C. E. Bugg, J. M. Thomas, M. Sundaralingam, and S. T. Rao, *Biopolymers*, 10, 175 (1971); (b) S. Arnott, *Progr. Biophys. Mol. Biol.*, 21, 265 (1970).
(3) S. Bram, *Nature (London), New. Biol.*, 232, 174 (1971).
(4) (a) C. D. Jardetzky and O. Jardetzky, *J. Amer. Chem. Soc.*, 82, 222 (1960); (b) C. D. Jardetzky, *ibid.*, 82, 299 (1960).
(5) M. P. Schweizer, A. D. Broom, P. O. P. T'so, and D. P. Hollis, *ibid.*, 90, 1042 (1968).
(6) P. O. P. T'so, N. S. Kondo, M. P. Schweizer, and D. P. Hollis, *Biochemistry*, 8, 997 (1969).
(7) S. S. Danyluk and F. E. Hruska, *ibid.*, 7, 1038 (1968).
(8) F. E. Hruska, A. A. Grey, and I. C. P. Smith, *J. Amer. Chem. Soc.*, 92, 4088 (1970).
(9) C. D. Barry, A. C. T. North, J. A. Glasel, R. J. P. Williams, and A. V. Xavier, *Nature (London)*, 232, 236 (1971).
(10) T. R. Emerson, R. J. Swan, and T. L. V. Ulbricht, *Biochemistry*, 6, 843 (1967).
(11) D. W. Miles, R. K. Robins, and H. Eyring, *Proc. Nat. Acad. Sci. U. S.*, 57, 1138 (1967); D. W. Miles, L. B. Towsend, M. J. Robins, R. K. Robins, and H. Eyring, *ibid.*, 93, 1600 (1971).
(12) W. Guschlbauer and Y. Courtois, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, 1, 183 (1968).
(13) W. Guschlbauer and M. Privat de Garilhe, *Bull. Soc. Chem. Biol.*, 51, 1511 (1969).
(14) (a) J. Donohue and K. N. Trueblood, *J. Mol. Biol.*, 2, 363 (1960); (b) A. E. V. Haschemeyer and A. Rich, *ibid.*, 27, 369 (1967).
(15) Previously, Schirmer, *et al.*, used the Overhauser effect to study a guanosine analog.[16a] A detailed discussion of molecular geometry determination by the Overhauser effect is given in their paper. There

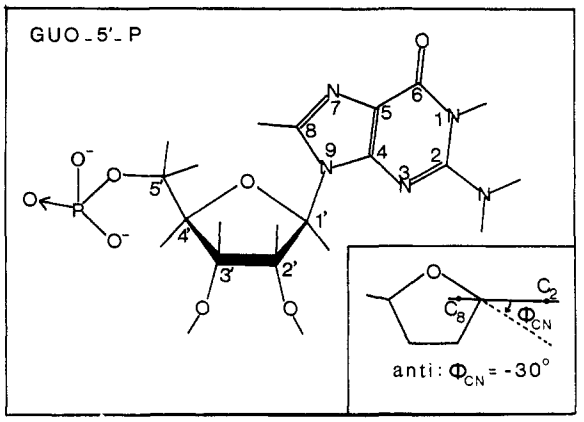

Figure 1. Molecular formula of Guo-5′-P. The molecule is shown in an anti conformation, in which the H_8 proton is closer to $H_{2'}$ and $H_{3'}$ than to $H_{1'}$. The 5′ protons are pictured pointing toward the base. H_8 is the only nonexchangeable proton on the base. The angle ϕ_{CN} is defined in the schematic view from above.

In this experiment one saturates a proton resonance and observes the resulting change in another proton's magnetization. In the case of dipole–dipole relaxation, the Overhauser enhancements give relative distances of the observed proton to the various saturated ones. Absolute distances, r, can also be obtained with the help of complementary relaxation measurements and simple assumptions concerning molecular movements. The available parameter is the *time average of* r^{-6}. An interesting point is that if the molecule is flexible, the computed proton–proton distances cannot be fitted to a single geometry and this will be evidence for the flexibility.

On space-filling models, one observes that the ribose proton closest to H_8 is $H_{1'}$ if the conformation is syn, and $H_{2'}$ or $H_{3'}$ if it is anti. This motivated us to measure the Overhauser enhancements of H_8 upon saturation of the various sugar protons. The interpretation of the inverse experiment, saturation of H_8 and observation of the sugar proton enhancements, is more complex, since the sugar protons also relax mutually between themselves.

A significant point of our study is that its object is a naturally occurring nucleotide in its natural solvent at concentrations where conclusions can be drawn concerning the isolated molecule. A novel method has been used to measure the Overhauser enhancements with high precision and sensitivity.

2. Experimental Section

Materials and Methods. The guanosine monophosphates (sodium salt) were obtained from P-L Biochemicals or Sigma. They were dissolved in D_2O and cleaned of divalent ions by shaking with Chelex-100 if necessary. After adjusting to pH ≈8, the samples were lyophilized twice and redissolved at final nucleotide concentrations of 0.025–0.15 *M*. Acid solutions were obtained by addition of concentrated DCl. The pD was taken as equal to measured pH plus 0.4,[18] the pH being measured with a Radiometer 28 pH meter. The solutions were purged of oxygen by washing with nitrogen for 15 min in the nmr tubes which were then capped and sealed with fast epoxy glue. Measurements were done within the following 2–3 hr.

It is well known that Guo-P's have a tendency to aggregate, and form gels within the pH range 2–6.[19] We used Guo-P concentrations as low as possible and we avoided the pH range of gel formation.[20]

The nmr spectra were recorded on a Varian HA-60 spectrometer in the frequency sweep mode. The lock was TMS contained in a capillary inside the sample tube.

In the measurement of the relaxation times, T_1, the line was first saturated by a strong radiofrequency (rf) field. The rf field was then turned off. Upon turning it up again after a given time, τ, the transient nutation[21] was observed in the absorption mode, and recorded on a fast Sanborn recorder. The variation of this signal as a function of τ gives T_1. With our values of T_1 (seconds), the sequences could be carried out manually.

To obtain the Overhauser enhancement, an audiofrequency generator was connected at the modulation input of the Varian 4311 rf unit. It was set at the frequency of the line to be irradiated. Two generators were connected when it was desired to saturate two lines simultaneously. Saturating fields were easily obtained; with an input voltage of 0.1 V (root-mean-square), attenuator setting 20 db, the rotating field measured by the frequency of transient nutation was 2.5 mG. The inverse enhancement was a linear function of the inverse squared voltage, as expected.[22]

The determination of Overhauser enhancements necessitates a precise measurement of signal intensity. If the signal is observed at low, nonsaturating rf fields, the intensity is proportional to the *area* under the peak. In principle the line width is unchanged during an Overhauser experiment; therefore the area and peak height are proportional, so that a peak height measurement would suffice. However, the proportionality fails if the line width varies. This may happen for two reasons. First, small, unresolved J couplings between the observed proton (I) and the saturated proton (S) might be decoupled by the saturating field. Second, the observed line width may be controlled by the field inhomogeneity and may then vary with time. These effects may be important, compared to enhancements which vary from below 5% up to a maximum of 50%.

We therefore operated as follows. The signal of proton I was observed in the dispersion mode (which can be recorded on the HA-60 after a minor modification), using a rf field which is large compared to the transverse relaxation time, T_2, possible unresolved J couplings, and inhomogeneity $\delta\omega$. Inspection of the Bloch equations shows that under these conditions[23] the signal line width is a function of the rf field, but the peak-to-peak *height* is proportional to $M_0\sqrt{T_2/T_1}$, where M_0 is the equilibrium magnetization and is therefore independent of the field inhomogeneity or unresolved J couplings. Since T_1 and T_2 are unaffected by saturation of proton S, the relative variation of the peak-to-peak height upon saturation of S is a direct measure of the Overhauser enhancement.

The signal observed in this way was perfectly reproducible, the precision on the height being limited only by the signal-to-noise ratio. Typically, using a sweep time across the line of 1 min, a precision of 2% was obtained, enabling us to determine Overhauser enhancements as small as 5% for sample concentrations of 5.10^{-2} *M*.

3. Spectral Assignments

The assignments of the H_8 singlet and the $H_{1'}$ doublet at neutral pD are taken from previous work.[4a,6] In the other cases, the assignments of lines in the same region are obvious. $H_{2'}$ is assigned by looking for the decoupling of the $H_{1'}$ doublet. $H_{3'}$ is a triplet due to coupling with $H_{2'}$ and $H_{4'}$. $H_{4'}$ is decoupled upon irradiating $H_{3'}$, and thus assigned. In turn, saturating $H_{4'}$ decouples the two 5′ protons ($H_{5'}$ and $H_{5''}$),

has been further work by the same group concerning other nucleosides and derivatives.[16b-d] The Overhauser effect is the object of ref 16e.

(16) (a) R. E. Schirmer, J. H. Noggle, J. P. Davis, and P. A. Hart, *J. Amer. Chem. Soc.*, **92**, 3266 (1970); (b) R. E. Schirmer, J. P. Davis, J. H. Noggle, and P. A. Hart, *ibid.*, **94**, 2561 (1972); (c) P. A. Hart and J. P. Davis, *ibid.*, **94**, 2572 (1972); (d) *ibid.*, **93**, 753 (1971); (e) J. H. Noggle and R. E. Schirmer, "The Nuclear Overhauser Effect: Chemical Applications," Academic Press, New York, N. Y., 1971.

(17) F. A. L. Anet and A. J. R. Bourn, *J. Amer. Chem. Soc.*, 87, 5250 (1965).

(18) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).

(19) J. F. Chantot, M. Th. Sarocchi, and W. Guschlbauer, *Biochimie*, **53**, 347 (1971).

(20) In the gel, a high resolution spectrum is still obtainable, demonstrating rapid movement. Line widths are of the order of 10 Hz.

(21) H. C. Torrey, *Phys. Rev.*, **104**, 563 (1956).

(22) A. Abragam, "The Principles of Nuclear Magnetism," Clarendon Press, Oxford, 1961, p 374.

(23) See ref 22, p 49.

thus completing and confirming the assignments. As an example, the spectra of Guo-2′-P are shown in Figure 2. Detailed analysis (at 60 and 250 MHz) of the chemical shifts and J couplings is left for a later publication.[24a] Here we note a number of systematic observations on the three guanine nucleotides. Similar observations have been made in earlier work,[5-7] but were restricted to a more limited range of compounds and of pD values, and to the exclusive observation of the H_8 and $H_{1'}$ protons.

(a) Upon lowering the pD, so that the base deuterates at N_7[24b] (pK = 2.2), H_8 shifts downfield by ~1 ppm, and $H_{1'}$ by ~0.2 ppm. Due to deuteration of the phosphate at the low pD, the sugar proton of the phosphoester bond also shifts downfield. The other protons are hardly affected. (b) Binding phosphate on a given sugar site shifts the corresponding proton downfield (~0.2 ppm). (c) The phosphorus–sugar proton J couplings at neutral pD are 4.5 Hz in Guo-5′-P (coupling to $H_{5'}$ and $H_{5''}$) and 7.0 Hz in Guo-2′-P (coupling to $H_{2'}$). We observed no coupling (<0.3 Hz) of phosphorus to more distant protons. (In Guo-3′-P, the $H_{3'}$ peak is hidden under HDO.) (d) In most cases the J couplings between the 5′ and 5″ protons give rise to a splitting, albeit barely resolved, showing that the chemical shifts of $H_{5'}$ and $H_{5''}$ are different.[24a] (e) Upon lowering the pD, there are changes in J, notably for Guo-5′-P where $J(1'-2')$ changes from 5.7 to 3.3 Hz. We feel that this change is too large to be explained by a charge effect due to protonation at N_7.[25] It is more probably due to a change in the dihedral angle[26] and therefore in the sugar conformation.[24a] (If the sugar is flexible the J values reflect time averages.)

4. The Overhauser Effect

4.1. The Two-Spin Case. Consider two magnetic dipoles I and S, for instance the nuclear magnetic moments of H_8 and $H_{1'}$, which are on the same molecule in solution. Each magnetic dipole creates a magnetic field $\mathbf{b}_{dip}$ at the other's location; the movement (rotation) of the molecule changes the orientation of the vector r linking the two magnetic dipoles, so that $\mathbf{b}_{dip}$ fluctuates in time. If the fluctuation spectrum has appreciable amplitude at the Larmor frequency ω, $\mathbf{b}_{dip}$ induces spin flips. This means that it can change the amplitude of the magnetization component parallel to the magnetic field; in other words, it contributes to the rate of longitudinal relaxation, $1/T_1$.

Detailed investigation of this problem leads to coupled equations for the longitudinal magnetizations M_I and M_S of I and S.[27] In the case of two protons, the magnetogyric ratios, γ_I and γ_S, of which are very close, one finds, in the absence of any rf field

$$\frac{d}{dt}M_I = -\rho_{IS}[(M_I - M_0) + 1/2(M_S - M_0)] \quad (1)$$

$$\frac{d}{dt}M_S = -\rho_{IS}[(M_S - M_0) + 1/2(M_I - M_0)] \quad (2)$$

(24) (a) Tran-Dinh Son, manuscript in preparation; (b) H. T. Miles, F. B. Howard, and J. Frazier, *Science*, 124, 1458 (1963).
(25) P. Laszlo and P. v. R. Schleyer, *J. Amer. Chem. Soc.*, **85**, 2709 (1963).
(26) M. Karplus, *J. Chem. Phys.*, **30**, 11 (1959).
(27) (a) I. Solomon, *Phys. Rev.*, **99**, 559 (1955); (b) A. Abragam, ref 22, p 333.

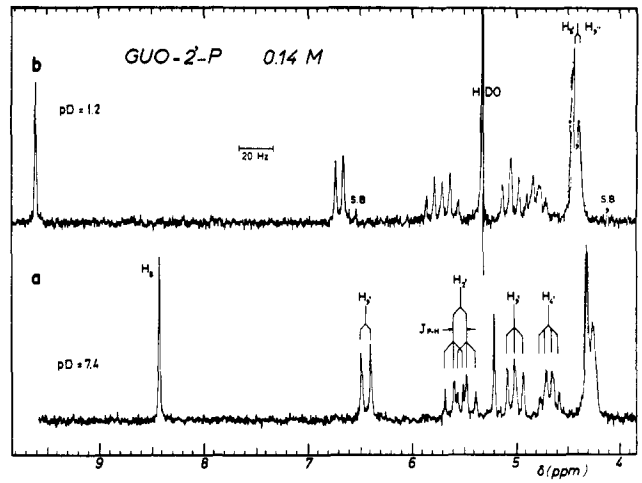

Figure 2. 60-MHz spectra of Guo-2′-P at 25°, 0.14 *M*: (a) pD 7.4; (b) pD 1.2. The large shift of H_8 is due to protonation at N_7. The chemical shifts are measured from a TMS capillary and are not corrected for bulk susceptibility.

where M_0 is the equilibrium magnetization of either species. If we introduce a strong rf field at the resonance frequency ω_S of S, we saturate the S resonance, so that $M_S = 0$. The steady-state solution of eq 1 is then $M_I = 1.5M_0$; the magnetization of I is enhanced by 50%. This is the steady-state Overhauser effect.

Equations 1 and 2 are derived under the following conditions: (a) the correlation time (τ_c) of the molecular rotation is short: $\omega\tau_c \ll 1$; (b) there are no other relaxation mechanisms than the dipole–dipole interaction between I and S. If either condition is not met, the enhancement of M_I on saturation of S will be smaller than +0.5.[28]

The value of ρ_{IS} is given by

$$\rho_{IS} = \left(\frac{\gamma^2\hbar}{4\pi\epsilon_0 c^2 r_{IS}^3}\right)^2 \tau_c \quad (3)$$

where γ is the magnetogyric ratio of the protons, r_{IS} their distance in meters, and $4\pi\epsilon_0 c^2 = 10^7$. For example, if $\tau_c = 10^{-10}$ sec and r_{IS} = 0.2 nm, ρ_{IS} = 0.9 sec^{-1}. Inspection of eq 1 shows that ρ_{IS}^{-1} is the relaxation time of M_I in an experiment where $M_S - M_0$ is maintained constant.[29] For instance, if at $t = 0$, $M_I = M_S = 0$ and if M_S remains zero at $t > 0$ due to saturation of S, then M_I varies according to

$$M_I(t) = 1.5M_0[1 - \exp(-\rho_{IS}t)] \quad (4)$$

In the two-spin case, the measurement of the "relaxation rate" ρ_{IS} enables one to determine the proton–proton distance by eq 3, if one knows the correlation time τ_c.

4.2. The Three-Spin Case. We consider now the case where the proton I is dipolar coupled to two spins S and T. Suppose I, S, and T are in the same rigid framework, the movements being isotropic. A straightforward extension of eq 1 gives

$$\frac{d}{dt}M_I = -\rho_{IS}[(M_I - M_0) + 1/2(M_S - M_0)] - \rho_{IT}[(M_I - M_0) + 1/2(M_T - M_0)] \quad (5)$$

(28) Relaxation by a scalar coupling between I and S would lead to a negative enhancement. This effect is negligible in the present study.
(29) Because the evolution of the magnetizations obeys a system of *coupled* equations (1, 2), the recovery of M_I is not exponential in the general case. Hence $1/\rho_{IS}$ is not, strictly speaking, a relaxation time.

We look again for steady-state solutions and for their physical interpretation. The simplest case is that where we saturate both the S and T resonances. Then we find that the steady-state value of M_I is again $1.5M_0$, and the "relaxation rate" is $\rho = \rho_{IS} + \rho_{IT}$. This is very similar to the two-spin case.

If we wish to obtain the distances r_{IS} and r_{IT} of I to S and T, we must determine ρ_{IS} and ρ_{IT} separately. A desirable way to do this would be to saturate the S resonance ($M_S = 0$) *while leaving* M_T *unperturbed* ($M_T = M_0$). The enhancement ξ_{IS} obtained easily from eq 5 would then be

$$\xi_{IS} = 0.5\rho_{IS}/\rho \tag{6}$$

One would obtain ξ_{IT} in a similar way. Substituting ρ_{IS} and ρ_{IT} by eq 3 then gives the ratio of distances from I to S and T

$$r_{IS}/r_{IT} = (\xi_{IS}/\xi_{IT})^{-1/6} \tag{7}$$

Lastly, the measurement of the "relaxation rate" ρ gives the absolute distances if one knows the correlation time τ_c. Note that the sum of the enhancements $\xi_{IS} + \xi_{IT}$ is still equal to 0.5.

However, if we are dealing strictly with a three-spin system, that is, if there are no other relaxation mechanisms than the couplings between the three spins (such as couplings to other spins), the saturation of the S resonance will *not* leave the magnetization of T unchanged, since T is relaxed both by I, the magnetization of which is now enhanced, and by S, the magnetization of which is null. In turn, the change in T's magnetization will change the enhancement of I. Such *indirect effects* are sensitive to the IST geometry. For instance, suppose T is between I and S, and closer to S, the configuration being I- -T-S. Due to the r^{-6} dependence, I is mainly relaxed by T which is mainly relaxed by S. Saturating S will enhance T's magnetization and this will reduce the magnetization of A below its equilibrium value; the enhancement of A is then *negative*. These indirect effects can be sorted out if the enhancements of both I and T are observed upon saturation of S.

4.3. The Overhauser Effect in the Presence of Other Relaxation Mechanisms. It was pointed out in section 4.1 that the Overhauser enhancement is reduced if other relaxation mechanisms are present. Among such mechanisms are interactions with paramagnetic species, or the effect of an anisotropic chemical shift, etc. There will be two effects of these extra relaxation pathways.

(a) Relaxation of Spin I. Equation 5 becomes

$$\frac{d}{dt}M_I = -\rho_{IS}[(M_I - M_0) + 1/2(M_S - M_0)] - \rho_{IT}[\ldots] - \rho_{other}(M_I - M_0) \tag{8}$$

where the last term expresses the other relaxation mechanisms. Then the enhancement is still given by eq 6, provided we take for ρ the value $\rho_{IS} + \rho_{IT} + \rho_{other}$. The meaning of this is simply that the enhancement is reduced by the existence of other relaxation mechanisms besides the dipolar coupling to the proton being saturated.

(b) Relaxation of Spins S and T. It was mentioned that saturation of S will change T's magnetization, giving rise to an indirect effect on I's magnetization. In the presence of extra relaxation mechansims for S and T, the relative importance of these effects is reduced since saturation of S now causes a smaller departure of M_T from M_0, whereas M_S is still null.

4.4. Principal Conclusions and Summary. (1) *From the ratio of enhancements,* ξ_{IS}/ξ_{IT}, *one derives the relative distances of the observed proton I to the saturated ones, S and T*

$$r_{IS}/r_{IT} = (\xi_{IS}/\xi_{IT})^{-1/6} \tag{7}$$

The absolute distances are obtained if one knows the relaxation rate $\rho = 1/T_1$ (where T_1 is the measured relaxation time) and the correlation time τ_c of the dipolar interaction (which is for instance computed by eq 10 below). According to eq 3 and 6, one has

$$r_{IS} = \left[\left(\frac{\gamma^2\hbar}{4\pi\epsilon_0 c^2}\right)^2 \frac{\tau_c}{\rho}\frac{0.5}{\xi_{IS}}\right]^{1/6}$$

or again

$$r_{IS} = 0.1\left(\frac{\tau_c}{10^{-11}}\frac{2.9}{\xi_{IS}\rho}\right)^{1/6} \tag{9}$$

where r_{IS} is in nanometers.

The above holds even in the presence of extra relaxation mechanisms, but assumes that there are no indirect effects.

(2) In the absence of indirect effects, if the sum of enhancements of a given proton (upon successive saturation of all its neighbors) is less than 0.5, other relaxation mechanisms are present, and/or $\omega\tau_c$ is not $\ll 1$.

(3) In practice the indirect effects may be quite small if the saturated proton is not the only relaxing agent of its neighbors, due to relaxation of these neighbors either by dipolar coupling to other protons or by other relaxation mechanisms. We found no evidence for indirect effects on the Overhauser enhancements of H_8 in the various Guo-P's.

5. Overhauser Effect in Guo-P

5.1. The Enhancements Are Incompatible with a Rigid Structure. We measured the enhancements of the H_8 signal upon saturation of the sugar proton resonances. In Table I we give the enhancements observed for Guo-2′-P, Guo-3′-P, and Guo-5′-P at 25°, in the neutral and acid forms, for solutions of low concentration.

The most important observation is that many protons contribute appreciable enhancements. Consider for example the case of Guo-5′-P at neutral pD. Let us take the inverse sixth power of the enhancement, which is proportional (eq 7) to the distance of each proton to H_8. For $H_{1'}$, $H_{2'}$, and $H_{3'}$, the numbers are 0.67, 0.62, and 0.69, respectively. It would seem that the distances of H_8 to these three protons are surprisingly similar. On a space-filling model, one can find a conformation ($\phi_{CN} \approx 0°$) which respects these relative distances, the absolute values being 0.4, 0.36, and 0.4 nm. However, the maximum distance that one can then contrive to one of the $H_{5'}$ and $H_{5''}$ protons is approximately 0.43 nm, which would lead one to expect an Overhauser enhancement, upon saturation of 5′ and 5″, of 12%, in contrast to 3.5% observed. Furthermore, we can estimate the relaxation rate of H_8, assuming these distances, by eq 9. The molecule is

Table I. Overhauser Enhancements of H_8 upon Saturation of the Sugar Protons $H_{1'}$, $H_{2'}$, $H_{3'}$, and the Two $H_{5'}$ Protons[a]

Nucleotide	pD	Concn, M	1′	2′	3′	5′ + 5″	Sum	T_1(sec) of H_8
Guo-2′-P	8.7	0.05	16	18.5	9.5	2.5	46.5	1.35
	1.2	0.05	16.5	8.0	4	4	32.5	1.45
Guo-3′-P	8.9	0.05	18	21		3.5	42.5	1.8
	1.4	0.05	15	13		5	33	1.9
Guo-5′-P	8.4	0.025	10.5	18	9.5	3	41	1.4
	1.3	0.05	7.5	9	14.5	7.5	38.5	2.3

[a] The enhancement upon saturation of $H_{4'}$ is always zero. The figures are obtained by extrapolation to complete saturation. They are given in per cent, and are good to ±2% or better. In the case of Guo-3′-P, $H_{2'}$ and $H_{3'}$ could be saturated only together. Note the large enhancement upon saturation of $H_{1'}$ compared to the other protons for Guo-3′-P at pD 1.2, and also the large number for $H_{3'}$ in Guo-5′-P at pD 1.3. The sum of the enhancements is nearly 50% for Guo-2′-P at neutral pD, and significantly less in the other cases.

roughly 1.1 × 0.8 × 0.5 nm. Using Stoke's formula for the rotational correlation time of a sphere of radius a

$$\tau_c = 4\pi\eta a^3/3kT \qquad (10)$$

where we take a = 0.4 nm, and $\eta = 10^{-3}$ Pa sec (the viscosity of water), we find $\tau_c = 6 \times 10^{-11}$ sec. Using the proton–proton distances just quoted, we find $\rho \approx 4 \times 10^{-2}$ sec^{-1}. This value is nearly 20 times smaller than the observed relaxation rate $\rho = 0.7$ sec^{-1}.[30]

The discrepancies between the predicted and the observed values of the enhancement due to $H_{5'}$, and of the relaxation rate, are significant arguments against the geometry presented above. Another argument is that it would be very surprising if H_8 were to position itself at distances from the sugar protons so exquisitely balanced. Furthermore, we would have to postulate as close a match for the distances in Guo-2′-P and Guo-3′-P, which also show enhancements from many sugar protons.

In summary, because of the r^{-6} dependence, the H_8 enhancement in a rigid molecule should come overwhelmingly from one proton, the one nearest to H_8. Therefore, the simple and straightforward observation of enhancements upon saturation of many protons is unexplainable in a rigid molecule; it gives strong evidence that *the molecule is flexible.* This conclusion is not dependent on precise measurements and/or interpretation of the relaxation time T_1.

We shall now show that with the assumption of a flexible molecule, the results are readily understood and yield a qualitative description of the intramolecular motion.

5.2. Analysis of the Enhancements for a Flexible Guo-P. In a flexible Guo-P, the distances of H_8 to the sugar protons will vary in time. The movement of primary concern here is rotation around the glycosidic bond. When $H_{1'}$ is closest to H_8 it will be the most important relaxing agent; later on, $H_{2'}$ will be closest and play the main role, etc. If the deformation of the molecule is slower than its rotation, the discussion of section 4 remains valid.[31] We need only replace r^{-6} in eq 3 by its time average $\langle r^{-6}\rangle$: the enhancement of H_8 upon saturation of a given ribose proton i will be proportional to $\langle r_i^{-6}\rangle$ where r_i is its distance to H_8. We can now easily explain the observed enhancements and relaxation rates. Because of the sixth power, the relaxation will occur mainly when H_8 is at its closest from some sugar proton, that is, for configurations where one r_i is close to the minimum value r_{min} that it achieves during the movement. In such configurations, *H_8 will be relaxed by only one proton at a time.* If p_i is the proportion of the time when $r_i \approx r_{i,min}$, one may write roughly

$$\langle r_i^{-6}\rangle \approx r_{i,min}^{-6} p_i \qquad (11)$$

with $\Sigma p_i \leqslant 1$. Previously we tried to fit the enhancement and relaxation data to eq 7 and 9 with distances r_i (from H_8 to the various sugar protons) which were invariable in time and therefore had to be geometrically compatible. We found that this was impossible because the simultaneous distances to the sugar protons were too large. For the flexible molecule, there is no compatibility condition on the $r_{i,min}$, and the data can be fitted easily.

In Table II we list (line 2) values of $r_{i,min} \times (p_i)^{-1/6}$ obtained from eq 9 and 11 using the data of Table I for Guo-5′-P at neutral pD, and a correlation time τ_c of 6×10^{-11} sec (eq 10). The minimum distances (on molecular models) and the corresponding molecular conformation are also shown (lines 4–6). The data under the heading "possible fit ..." (line 3) are derived from the Overhauser enhancements (line 1) with the choice of p indicated, where p is the proportion of the time spent in each of the three suggested conformations. We see that it is possible to find p values such that the derived r_{min} compare favorably with those from the molecular model (line 4). Furthermore, $\Sigma p_i = 1$, and this shows that the molecule spends little time in orientations (for instance, $\phi_{CN} = -10$ to $+60°$) for which H_8 is far from all sugar protons. This conclusion would hold also for the other cases of Table I, since the total enhancements and the relaxation rates are similar.

5.3. Syn *vs.* Anti. From the above analysis, we can define two classes of orientations, those for which the distance of H_8 to $H_{1'}$ is near its minimum ($\phi_{CN} \approx +120°$), and those for which the distance of H_8 to either $H_{3'}$ or $H_{2'}$ is near its minimum ($\phi_{CN} \approx -70°$ and $-120°$). In the case of Guo-5′-P at neutral pD, the probabilities of these two classes are equal to: 0.5 in the first case, 0.2 + 0.3 in the second. The angles describing the two classes are not far from those used by Haschemeyer and Rich[14b] to define the syn and anti ranges: $\phi_{CN} = +150 \pm 30°$ for syn, and $\phi_{CN} = -30$

(30) The difference would be smaller if we used a larger τ_c value for the computation of the relaxation rates. A larger τ_c would occur if the molecules were aggregated, but aggregates of more than two molecules are expected to be rare in our conditions (J. F. Chantot, private communication). See also section 5.4.

(31) A supplementary condition for this result is that the deformation should be fast compared to the observed proton's relaxation time T_1. Otherwise the Overhauser effect is modified. This is the "slow case" which is treated in ref 16b and 16c. In the "slow case," relaxation will usually not be exponential, a feature of great diagnostic value which was not pointed out previously. In our experiments, the relaxation is always exponential, so that we can disregard the "slow case," *i.e.* the movement is faster than ≈1 sec^{-1}.

Table II. Distances of H_8 to $H_{1'}$, $H_{2'}$, and $H_{3'}$ in Guo-5'-P[a]

	$H_{1'}$	$H_{2'}$	$H_{3'}$
1. Overhauser enhancement, %	10.5	18	9.5
2. $\langle r^{-6}\rangle^{-1/6} \approx r_{min}p^{-1/6}$, nm	0.25	0.22	0.25
3. Possible fit of $r_{min}p^{-1/6}$			
(a) p	0.5	0.3	0.2
(b) r_{min}, nm	0.22	0.18	0.19
4. Minimum distance from molecular models (nm), for the best choice of ϕ_{CN}, and allowing			
(a) only one sugar conformation	0.23	0.19	0.21
(b) the most favorable sugar conformation in each case	0.23	0.18	0.19
5. Value of ϕ_{CN} for minimum distances obtained in lines 4a and 4b, deg	+120	−120	−70
6. The corresponding conformation seen from above (schematic)			

[a] At neutral pD and $T = 25°$ as derived from the Overhauser enhancements and the relaxation time, using the description for a flexible molecule given in the text, and a correlation time $\tau_c = 6 \times 10^{-11}$ sec. The derived minimum distances (nm) from H_8 to the proton indicated at the column head appear in line 3b. In line 4 they are compared to the minimum distances taken from molecular models. The sugar conformation in line 4a has the 3'-CH bond slightly more axial than the 2'-CH bond. The drawings indicate the conformations in which the minimum distance to the given sugar proton is obtained; the base (its plane perpendicular to the paper) is drawn as an arrow with H_8 at its tail. The derived r_{min} values compare favorably with those from the molecular model, although the latter tend to be slightly larger.

± 30° for anti. We shall therefore describe as syn the orientations where H_8 is close to $H_{1'}$ and anti those where H_8 is close to $H_{2'}$ or $H_{3'}$. Guo-5'-P shares its time equally between these two classes of conformations.

Rather than go through the same analysis for the other Guo-P's, we shall now give a simplified formula for the probabilities of these conformations. In eq 7 and 11, we observe that the probability p is proportional to $\xi(r_{min})^{-6}$. *We now take* r_{min} *from the molecular models* (Table II, lines 4a and 4b), whence we obtain: $[r_{min}(H_{2'})]^6 \approx [r_{min}(H_{3'})]^6 \approx (1/3)[r_{min}(H_{1'})]^6$. A given enhancement by $H_{1'}$ reflects more of a syn conformation than the anti character corresponding to the same enhancement by $H_{2'}$ or $H_{3'}$ because the minimum possible distance to $H_{1'}$ is greater.[32] We therefore suggest as a qualitative measure

$$p(\text{syn}) = \frac{3\xi(H_{1'})}{3\xi(H_{1'}) + [\xi(H_{2'}) + \xi(H_{3'})]} \qquad (12)$$

$$p(\text{anti}) = 1 - p(\text{syn})$$

The results of this crude evaluation are given in Table III for all the Guo-P's. Although the numerical values should be taken with caution, and although the geometrical definitions of syn and anti conformations are loose, the table gives strong indications of a large proportion of syn conformations. Moreover, this proportion varies according to compound and conditions. Noteworthy are acid Guo-2'-P and Guo-3'-P which are mostly syn. Guo-5'-P is statistically less syn than the other monophosphates. This may be because the syn conformation is favored by temporary $N_3\cdots H\text{–}O_{5'}$ hydrogen bonding in the 2' and 3' compounds.[33]

Table III. Proportion of the Time p(syn) Spent in Syn Conformations[a]

	pD	p(syn)	p(syn)/p(anti)
Guo-2'-P	8.7	0.63	1.7
	1.2	0.80	4.0
Guo-3'-P	8.9	0.72	2.6
	1.4	0.78	3.5
Guo-5'-P	8.4	0.53	1.1
	1.3	0.49	1.0

[a] As given by eq 12. As used here syn means ϕ_{CN} in the region of +120°, anti means ϕ_{CN} in the region of −70 to −120°. Note that p(anti) = 1 − p(syn); $T = 25°$.

5.4. Further Considerations. (a) We have studied the Overhauser enhancements of H_8 as a function of temperature between 5.5 and 44°. Table IV shows the results for Guo-2'-P at pD 7.4, 0.1 *M*. There is no drastic change in the relative values of the enhancements *vs.* temperature, indicating that the distribution between the various molecular conformations does not change much with temperature, from which we conclude that the energy difference between the conformational minima is small. It is furthermore possible that the intramolecular motion does not involve a large activation energy. One may then expect the movement to be rather fast, with no strongly preferred conformation. On the other hand, some conformations are sterically hindered, as can be seen on molecular models. For instance, H_8 and $H_{2'}$ may come within their van der Waals distance, as may also the phosphate and amino groups in Guo-5'-P, etc. Experimentally, the rate of the movement is not directly ac-

Table IV. Overhauser Enhancements of H_8 as a Function of Temperature in Guo-2'-P[a]

T, °C	1'	2'	3'	5' + 5''	Sum
5.5	14	14.5	9	1	38.5
25	13	19	4	2	37
44	18	21	6.5	2	47.5

[a] pD 7.4, 0.1 *M*. The relative values are nearly independent of temperature. The enhancement sum increases with temperature. Similar results were obtained with the other Guo-P's.

(32) One could attempt to treat $H_{2'}$ and $H_{3'}$ differently, taking into account the sugar conformation. Such a refinement seems unwarranted at this point.

(33) S. T. Rao and M. Sundaralingam, *J. Amer. Chem. Soc.*, 92, 4963 (1970).

cessible; we can only state that it is larger than the proton relaxation rate ($\sim$1 sec^{-1}), since otherwise we would be dealing with a mixture of different molecules on the time scale of the experiment, and the relaxation would not be exponential, contrary to observation.[34]

(b) For most cases of Table I where they can be obtained separately, $\xi(H_{2'})$ is larger than $\xi(H_{3'})$. In the picture of a more or less free rotation, suggested above, the ξ values reflect the minimum distances; hence we conclude that the minimum distance of H_8 to $H_{3'}$ is larger than to $H_{2'}$, as indicated in Table II ("one sugar conformation"). This suggests that $C_{3'}$–$H_{3'}$ is not close to the axial direction. An exception is Guo-5′-P at low pD where $\xi(H_{2'}) < \xi(H_{3'})$; this must indicate a change of the sugar geometry, in which $C_{3'}$–$H_{3'}$ becomes closer to axial, or $C_{2'}$–$H_{2'}$ further from axial. The second possibility is suggested by analysis of the J couplings which indicate that $C_{2'}$–$H_{2'}$ takes a more equatorial direction when the pD is lowered.[24a] The fact that the relaxation time increases in Guo-5′-P at low pD also supports an increase of the H_8–$H_{2'}$ distance rather than a decrease of the H_8–$H_{3'}$ distance.

(c) The $H_{5'}$ enhancements of Table I are small but not equal to zero, in contrast with those of $H_{4'}$ which are always zero. Taking due account of the fact that there are two protons, $H_{5'}$ and $H_{5''}$, the values of $\langle r^{-6}\rangle^{-1/6}$ derived from the enhancements are in the neighborhood of 0.32 nm. For H_8 to come that close to $H_{5'}$ it is necessary that most of the time the N_9–$C_{1'}$ bond be not far from axial to the sugar (Figure 1) rather than equatorial, and also that one of the $C_{5'}$–$H_{5'}$ vectors point toward the base, at least part of the time.

(d) We examined the Overhauser enhancements as a function of nucleotide concentration. Typical results are shown in Table V. The most striking change is the relative diminution of the $H_{1'}$ enhancement, as the concentration increases. Our interpretation is that aggregation occurs at the higher concentrations, and that in the aggregates, an anti conformation is favored. The conformation observed in Guo-5′-P crystals is also anti.[36]

Table V. Overhauser Enhancements of H_8 *vs.* Concentration in Guo-5′-P[a]

Concn, M	1′	2′	3′	5′ + 5″	Sum	T_1, sec
0.025	10.5	18	9.5	3	41	1.4
0.05	7	19.5	11	4.5	42	1.3
0.1	4.5	16	8	4	32.5	1.1

[a] pD 8.4, T = 25°. The relative enhancement due to $H_{1'}$, the enhancement sum, and T_1 are smaller at high concentrations. These effects are due to aggregates in which the anti form is favored. Similar results are obtained in Guo-3′-P. There is little aggregation in Guo-2′-P.

5.5. Relaxation. Previously we used the relaxation times for the evaluation of absolute distances only; most of the conclusions reached above were dependent mainly on the *ratios* of the Overhauser enhancements. Here we shall be concerned with the value of the *sum* of the enhancements, and the fact that it is in some cases significantly smaller than the theoretical maximum of 0.5; there is an "enhancement defect" (Table I).

According to the discussion of section 4 (conclusions in 4.4), this could mean that there are extraneous relaxation mechanisms. However, these mechanisms would have to be rather efficient; if the sum of Overhauser effects is 30% instead of 50%, with a total relaxation rate ρ of 0.5 sec^{-1}, the extraneous rate ρ_{other} is given by $\rho_{other}/\rho = (50 - 30)/50$, whence $\delta_{other} = 0.2$ sec^{-1}. Remaining dissolved oxygen or paramagnetic ions seem unable to account for such a rate and indeed the enhancements were not affected by the extent of degassing nor by addition of the chelating agent EDTA. Relaxation by dipolar coupling to the nitrogen nuclei is also negligible; we compute it by eq 3 as $\rho \approx 10^{-3}$ sec^{-1}. Modulation of the proton–nitrogen scalar coupling by quadrupolar relaxation of the nitrogens would only affect the proton T_2, not T_1.

On the other hand, we observed a rather systematic increase of the enhancement sum, together with an increase in T_1, upon raising the temperature or diminishing the concentration, as seen, for example, in Tables IV and V. This suggests that the enhancement sum is reduced in conditions which favor aggregation. Another observation in favor of this proposition is that the enhancement defect is larger in the acid Guo-P's (Table I) where some aggregation could be expected since the experiments were carried out only 1.0 pD unit below the gel formation pK.

Aggregation could bring about an enhancement defect if the intermolecular H_8–H_8 distances in the aggregates were small; the H_8–H_8 dipolar interaction would cause relaxation but no Overhauser enhancement of H_8.[37] We disproved this hypothesis by an experiment in which we exchanged half of the H_8 for deuterium[5,38] (15 min at $T \approx 70°$); this caused no increase in the enhancement sum.

Another possible effect of aggregation would be to increase the rotation correlation time τ_c to values so large that $\omega\tau_c$ is not much smaller than 1. Formulas 1, 2, and 3 are then modified in such a way that the maximum enhancement ξ_{max} is smaller than 0.5. For instance, if $\omega\tau_c = 0.5$, ξ_{max} is only 0.31, while for $\omega\tau_c = 1$, corresponding to $\tau_c = 2.5 \times 10^{-9}$ sec, ξ_{max} falls to 0.05. Note that this τ_c value is 40 times larger than that corresponding to the isolated nucleotide. Such a τ_c would be expected if the nucleotide were part of a large aggregate containing perhaps up to 40 nucleotides. The interesting point is that if each nucleotide were part of such an aggregate during *only 2% of the time*, exchanging rapidly ($<10^{-2}$ sec) between the aggregate and the solution, this would increase the relaxation rate by 29% over the rate for the free nucleotide, and correlatively reduce the enhancement sum from 0.50 to 0.38 (see Appendix). This could very well explain the observed enhancement defect. Fugitive aggregates of this sort, involving at any time only a few per cent of the total number of nucleotides, would pass unnoticed in most investigations of aggregation.[39]

(34) Evidence for a rapid syn–anti process (10^{-9} sec) is provided by recent ultrasonic relaxation experiments.[35]

(35) L. M. Rhodes and P. R. Schimmel, *Biochemistry*, **10**, 4426 (1971).

(36) W. Murayama, N. Nagashima, and Y. Shimizu, *Acta Crystallogr., Sect. B*, **25**, 2236 (1969).

(37) Intermolecular relaxation of H_8 by, *e.g.*, $H_{1'}$ would contribute normally to the Overhauser effect.

(38) F. J. Bullock and O. Jardetzky, *J. Org. Chem.*, **29**, 1988 (1964).

(39) J. F. Chantot, private communication.

5.6. Comparison with Related Work. In a series of studies,[16a-d] the 2′,3′-isopropylidene derivatives of a number of nucleosides have been investigated by the Overhauser effect, as well as cytidine, uridine, and halogen derivatives of uridine. In many cases the conclusion was drawn that the solutes exhibit a range of conformations, a conclusion similar to that which we here reach for the case of the Guo-P's.

It may be worthwhile to point out a difference of philosophy between the above-mentioned studies and ours. In the former, there has been considerable effort to develop an elaborate theoretical picture[16e] of the Overhauser effect, notably as regards flexible molecules. Based on this work and on the fitting of the experimental data, a refined model of the molecular motion is proposed, involving typically two gaussian distributions of the glycosidic angle ϕ_{CN}, whose centers and widths are adjusted. As for us, we have felt that we could not refine the interpretation to that extent. because our knowledge of the geometry and of the various modes of internal motion of the Guo-P's is insufficient. Rather, we have strived to make the model as simple as possible, and to use all possible experimental data (in particular the relaxation time) to provide a very strong footing for a number of crucial conclusions, notably the flexibility of the molecule.

6. Conclusions

The principal results of this study are the demonstration of the flexibility of the guanosine monophosphates, and the evaluation of the distribution between the syn and anti conformations. As shown in Table III, the isolated Guo-P's spend a large proportion of the time in syn conformation when in solution. There is no change in this distribution with temperature, suggesting a relatively free and probably fast movement between the syn and anti conformations. Syn conformations are favored at low pD. Upon aggregation the structure becomes more anti; this makes sense since the preferred structures in crystals and in polynucleotides are also anti.

Our observations should be compared with CD studies. Mobility of the base with respect to the sugar was suggested on the basis of the weakness of the CD[13] and this is borne out in the present study. On the other hand the inversion of CD, which occurs in all guanosine monophosphates upon protonation,[40] does not seem to correlate simply with the syn *vs.* anti parameter (Table III) whose sensitivity to pD varies widely among the different monophosphates.

In this study we also show that the N_9–$C_{1'}$ bond is not far from axial to the sugar, and information is obtained regarding the sugar conformations of the various monophosphates. These results will be completed by the analysis of high-field spectra to yield a description of the geometry and degree of rigidity of the sugar–phosphate moiety.[24a]

The analysis of relaxation rates is an indispensable complement to the enhancement data. Here it was involved in the demonstration of molecular flexibility (section 5.1), while the search for relaxation mechanisms explaining the "enhancement defect" (section 5.4) suggests the presence of fugitive aggregates. The Overhauser enhancement defect is shown to be a powerful probe of such aggregates, which would be difficult to study by other methods.

Lastly, we feel that the present work demonstrates the possibility of applying the Overhauser effect and relaxation methods to systems as large and complex as a flexible mononucleotide, using no more than simple and straightforward models.

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7. Appendix

Overhauser Effect in the Presence of Aggregates. We compute the Overhauser enhancement of H_8 for a nucleotide exchanging between one environment where $\omega\tau_c \ll 1$ (isolated nucleotide) and another one where $\omega\tau_c \ll 1$ (large aggregate). We consider the two-spin case for simplicity.

The general formula for the evolution of M_I[27] is

$$\frac{d}{dt}M_I = \rho(M_I - M_0) - \sigma(M_S - M_0) \qquad (A1)$$

which replaces eq 1. Upon saturation of S ($M_S = 0$), the steady-state solution corresponds to an enhancement, $\xi = \sigma/\rho$. The equations for ρ and σ are

$$\rho = K\tau_c\left(2 + \frac{6}{1 + \omega^2\tau_c^2} + \frac{12}{1 + 4\omega^2\tau_c^2}\right) \qquad (A2)$$

$$\sigma = K\tau_c\left(-2 + \frac{12}{1 + 4\omega^2\tau_c^2}\right) \qquad (A3)$$

where K is a constant. σ/ρ is 0.5 for $\omega\tau_c \ll 1$, and diminishes rapidly as $\omega\tau_c$ becomes close to 1, and larger.

If the lifetime in each environment is long compared to the corresponding τ_c, eq 1 remains valid at any given time, with the values of ρ and σ given by eq A2 and A3, which now vary in time, as the nucleotide exchanges between one environment and the other. Furthermore, if the exchange rate is fast compared to the relaxation rates,[41] we can take a time average of eq A1. Hence the steady-state solution is given by

$$\langle\rho\rangle(M_I - M_0) - \langle\sigma\rangle(M_S - M_0) = 0$$

The "relaxation rate" is $\langle\rho\rangle$ and the enchancement ξ is $\langle\sigma\rangle/\langle\rho\rangle$.

Numerical Example. $\tau_{c_1} = 6 \times 10^{-11}$ sec, during 98% of the time: $\tau_{c_2} = 2.5 \times 10^{-9}$ sec, during 2% of the time. Using eq A2 we compute

$$\rho_2 = \rho_1\frac{7.4(2.5 \times 10^{-9})}{20(6 \times 10^{-11})} = 15.5\rho_1$$

$$\therefore \langle\rho\rangle = 0.98\rho_1 + 0.02\rho_2 = 1.29\rho_1$$

Hence the relaxation rate changes by less than one-third. The average Overhauser enhancement is, eq A2 and A3

$$\xi = \frac{\langle\sigma\rangle}{\langle\rho\rangle} = \frac{0.98 \times 10 + 0.02 \times 0.4 \times (2.5 \times 10^{-9}/6 \times 10^{-11})}{0.98 \times 20 + 0.02 \times 7.4 \times (2.5 \times 10^{-9}/6 \times 10^{-11})} = \frac{9.8 + 0.03}{2 \times 9.8 + 6} = 0.38$$

It is reduced significantly below 0.50.

(40) W. Guschlbauer, unpublished.

(41) As in section 5.4 this assumption is justified by the observation that the relaxation is exponential.

In words: during the small part of the time in which the molecule is in an aggregate, it relaxes much faster (15 times) than the monomer, and during that time it relaxes toward the little enhanced steady-state magnetization $M_0 \times 1.05$. The effects of the aggregation are on one hand to raise the average relaxation rate by ~30% and on the other to decrease the Overhauser enhancement by ~20%, relative to the respective values for the case of a never-aggregated molecule. But, whereas there are no "never-aggregated" data with which to compare the observed relaxation time, the effect is clearly visible on the Overhauser enhancement, because its value for the never-aggregated case is known to be exactly 0.5.

Reaction of the Carbanionic Aldolase–Substrate Intermediate with Tetranitromethane. Identification of the Products, Hydroxypyruvaldehyde Phosphate and D-5-Ketofructose 1,6-Diphosphate[1]

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Abstract: The reaction of the carbanion–enamine intermediate of the aldolase dihydroxyacetone phosphate complex ($^-CHOHC{=}NH^+RCH_2OPO_3^{2-}$; H_2NR = lysyl residue of aldolase) with tetranitromethane resulted in irreversible modification of the substrate. The nature of the reaction has now been elucidated by isolation and identification of the products. The products were separated by anion exchange chromatography and their structures deduced from their chromatographic and spectral properties and from their specificity as substrates in enzymatic analyses. The effect of tetranitromethane is an oxidation of the carbanion–enamine intermediate of dihydroxyacetone phosphate to hydroxypyruvaldehyde phosphate ($CHOCOCH_2OPO_3^{2-}$), the reagent being reduced to nitrite and nitroformate. A secondary product is generated in an aldolase-catalyzed condensation of hydroxypyruvaldehyde phosphate with dihydroxyacetone phosphate, yielding D-5-ketofructose 1,6-diphosphate. The occurrence of specific oxidation of aldolase-activated dihydroxyacetone phosphate at C-3 thus accounts for the trapping of the carbanion by tetranitromethane. The susceptibility of the carbanion to oxidation may indicate a general applicability of suitable oxidants as mechanistic probes of nonenzymatic and enzymatic reactions involving carbanionic intermediates.

Intermediates of enzymatic reactions can be detected and identified on the basis of the specific reactivity which certain groups of the enzyme–substrate complex acquire transiently in the course of catalysis. Such syncatalytic reactivity changes may involve certain groups both of the enzyme and of the substrate moiety of the enzyme–substrate complex.[2] The latter possibility has been exemplified by an intermediate in the reaction mechanism of fructose 1,6-diphosphate aldolase which was found to be selectively reactive toward tetranitromethane.[3,4]

The aldolase-catalyzed cleavage–condensation reactions are thought to involve an intermediary carbanion on C-3 of dihydroxyacetone phosphate which is evidenced by stereospecific pro-*S* hydrogen isotope exchange on C-3 in the absence of an aldehyde.[5,6] In class I aldolases the intermediary carbanion apparently is stabilized by resonance with a protonated Schiff base of C-2 to an active-site lysyl ε-amino group;[7] in class II aldolases it is stabilized by interaction with a zinc atom at the active site.[8–10] Kinetic examination of the reaction of tetranitromethane with the aldolase–substrate intermediate as well as the selective reactivity of the reagent toward the carbanionic forms of carbon acids[11] indicated a carbanion trapping action of the reagent. Tetranitromethane reacts also with a number of catalytic systems other than aldolase which are thought to involve carbanionic intermediates, *i.e.*, pyridoxal plus glutamate,[3] aspartate aminotransferase plus the substrate analog *erythro*-β-hydroxyaspartate,[12] and the substrate complexes of pyruvate decarboxylase and of 6-phosphogluconate dehydrogenase.[13]

During the reaction of the ternary system of aldolase, substrate, and tetranitromethane the concentration of substrate was found to decrease progressively, thus indicating that the substrate was modified irreversibly.[3] In the present study the substrate derivative produced in the reaction of tetranitromethane with the aldolase-

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(2) P. Christen, *Experientia*, **26**, 337 (1970).
(3) P. Christen and J. F. Riordan, *Biochemistry*, 7, 1531 (1968).
(4) J. F. Riordan and P. Christen, *ibid.*, 8, 2381 (1969).
(5) I. A. Rose, *Brookhaven Symp. Biol.*, **15**, 293 (1962).
(6) J. F. Biellmann, E. L. O'Connell, and I. A. Rose, *J. Amer. Chem. Soc.*, **91**, 6484 (1969).
(7) B. L. Horecker, P. T. Rowley, E. Grazi, T. Cheng, and O. Tchola, *Biochem. Z.*, **338**, 36 (1963).
(8) W. J. Rutter, *Fed. Proc.*, **23**, 1248 (1964).
(9) R. D. Kobes, R. T. Simpson, B. L. Vallee, and W. J. Rutter, *Biochemistry*, **8**, 585 (1969).
(10) A. S. Mildvan, R. D. Kobes, and W. J. Rutter, *ibid.*, 10, 1191 (1971).
(11) P. Christen and J. F. Riordan, *Anal. Chim. Acta*, **51**, 47 (1970).
(12) S. V. Shlyapnikov and M. Y. Karpeisky, *Eur. J. Biochem.*, 11, 424 (1969).
(13) M. J. Healy and P. Christen, *Experientia*, 28, 736 (1972); *Biochemistry*, in press.