Nanosecond Relaxation Processes in Aqueous Mononucleoside Solutions*

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ABSTRACT: Relaxation spectra of aqueous mononucleoside solutions have been investigated by an ultrasonic relaxation method in the frequency range of 10–250 MHz. Experiments were conducted under conditions where effects of base ionization reactions are negligible. Solutions of adenosine show a single, concentration-independent relaxation process having a time constant of 2.9 nsec at 36°. Several lines of evidence rule out the possibility that the relaxation process arises from solvation of the nucleoside. For example, neither purine nor ribose by itself exhibits any relaxation effect. In addition, the relaxation process is not due to ribose ring puckering equilibria since the effect is present in 2′,3′-*O*-isopropylideneadenosine, a nucleoside in which mobility of the ribose ring is greatly restricted. By elimination of solvation and ribose puckering equilibria, together with other lines of evidence, it is concluded that the relaxation process probably arises from the syn–anti transition of the glycosidic bond. The temperature dependence of the adenosine relaxation time yields an apparent activation energy of 6.2 kcal mole^{-1}, in reasonable agreement with some of the recent calculations of barrier heights to rotation in adenosine. The relaxation time for 2′-deoxyadenosine at 25° is about a factor of 2 shorter than that for adenosine. This might be explained by the fact that the 2′-OH can conflict sterically with the adenine ring when the syn–anti transition occurs with a 2′-endo pucker of ribose. Relaxation processes have also been observed and characterized for guanosine, inosine, and formycin. In the case of pyrimidines, no effect is observable with cytidine and with uridine an incipient small effect is present which falls below the frequency range accessible to us. The absence of processes similar to the purine nucleosides is not surprising in view of the evidence which suggests that pyrimidines are predominantly anti.

Mononucleoside units participate in several types of interactions and structural rearrangements which play an important role in biasing nucleic acid configurations toward certain conformations. Furthermore, the dynamic aspects of nucleic acid structural and shape changes can certainly be understood in part in terms of the kinetic behavior of the individual mononucleoside units. Of obvious importance are the intermolecular pairing and stacking reactions of the individual bases. Significant unimolecular processes undergone by the nucleosides include the syn–anti transition (Donahue and Trueblood, 1960) of the glycosidic bond and the various endo–exo puckerings of the ribose moiety.

These rearrangements and reactions of mononucleosides are elementary processes which may be expected to occur with great rapidity, so that specialized techniques are required in order to investigate them on a kinetic basis. For example, Hammes and Park (1968, 1969) succeeded in studying the dynamics of hydrogen bonding between certain mononucleosides and nucleoside analogs in nonaqueous solvents by using an ultrasonic relaxation method. In their studies, time constants of the order of 10^{-8}–10^{-9} sec were found for the hydrogen-bonding reactions. Similar time constants might be expected for other reactions of mononucleosides.

Some time ago an investigation of the various possible reactions and structural transitions of mononucleosides in aqueous solvents was undertaken in this laboratory. The use of aqueous systems precludes the intermolecular association *via* hydrogen bonding, thus enabling attention to be focused on some of the other processes enumerated above. Using the ultrasonic technique (Herzfeld and Litovitz, 1959), a unimolecular relaxation process for adenosine was observed which has a time constant of a few nanoseconds. Many other nucleosides were subsequently investigated and some of them were also found to undergo relaxation processes in the nanosecond time range. From investigations of the relaxation behavior of these molecules, it is concluded that the syn–anti interconversion of the glycosidic bond is the molecular process which best explains all of the data. These rotational isomers are roughly indicated for adenosine in Chart I. A variety of experiments with many of the compounds listed in Chart I, together with other lines of evidence, have ruled out other possible unimolecular processes. The results obtained show that the purine nucleosides have roughly comparable relaxation times as might be expected on the basis of their similar structures. The pyrimidines cytidine and uridine do not have relaxation processes in the same time range although an incipient relaxation effect with relatively high concentrations of uridine is observed at low (~20 MHz) frequencies. This difference in behavior between purine and pyrimidine nucleosides is consistent with the predictions that the pyrimidines exist in predominantly the anti form, whereas purines can more readily adopt both syn and anti conformations (Tinoco *et al.*, 1968; Lakshminarayanan and Sasisekharan, 1970).

Experimental Section

All chemicals used were of the highest purity available. Adenosine, deoxyadenosine, guanosine, uridine, D-ribose, inosine, and adenosine 5′-monophosphate were obtained

* From the Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received July 26, 1971.* This work was supported by Grant GM-15539 from the National Institutes of Health, an award from the Alfred P. Sloan Foundation, and M.I.T. resources.

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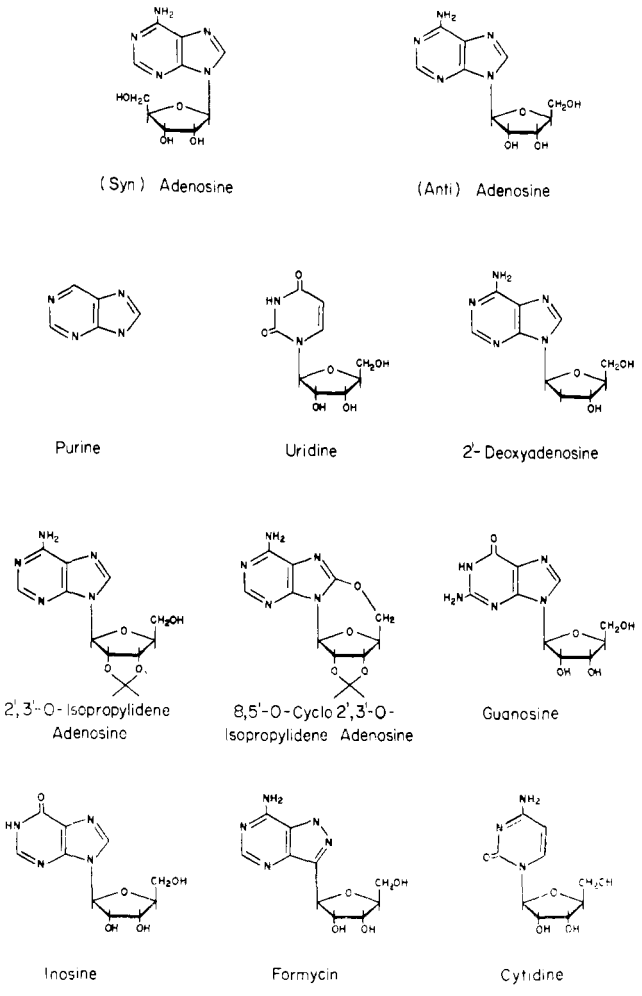
CHART I: Structures of Nucleosides and Nucleoside Derivatives



from Calbiochem (A grade). The nucleoside 2′,3′-*O*-isopropylideneadenosine was obtained from Sigma Chemical Co. and from Calbiochem, both preparations being homogeneous when chromatographed on cellulose thin-layer sheets in two different solvent systems. Cytidine and ribose 5-phosphate were obtained from Sigma Chemical Co. and purine was purchased from Cyclo Chemical Co. Formycin was the gift of Dr. H. Umezawa and Dr. T. Wakazawa of Meiji Seika Kaisha, Ltd., and was found to be homogeneous by cellulose thin-layer chromatography (R_F 0.62 in isopropyl alcohol–water, 7:3, v/v). Urea (Baker analyzed reagent grade) was used without further purification. Water used for all experiments was distilled, deionized with a mixed-bed resin, and redistilled in a Corning AG-3 all glass still.

Constant temperatures were maintained with a Lauda/Brinkmann Model K-2/R circulator. The temperature within the ultrasonic measurement cell was determined using a copper-constantan thermocouple. A Radiometer pH meter equipped with a GK 2021 C electrode was used to measure the apparent pH.

The procedure for determining the pressure amplitude absorption coefficient, α, and the ultrasonic sound velocities was similar to that described in detail elsewhere (Burke *et al.*, 1965; Lewis, 1965). A Matec model 6000 pulse generator and receiver equipped with the Model 960 plug-in unit was employed for attenuation measurements in the frequency range of 10–250 MHz. The amplitude of the ultrasonic pulse was

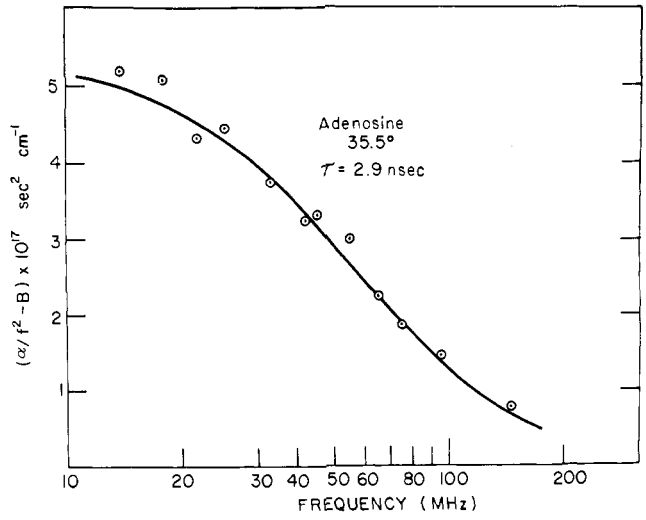


FIGURE 1: Plot of $(\alpha/f^2) - B$ *vs.* f for a solution of 0.046 M adenosine in H_2O, 35.5°. The curve is calculated by eq 1 using parameters given in Table I.

measured with a Matec Model 1235A pulse amplitude monitor, having an accuracy of ±0.1 db. A General Radio Corp. Model 1591-AR line voltage regulator was used to maintain the output of the high-frequency pulse generator at a constant level.

The absorption coefficients were measured to an accuracy of better than ±2% except at low frequencies (<15 MHz) where the decreased attenuation of the solutions caused slightly larger deviations. The sound velocity was measured to better than ±1%.

Results and Treatment of Data

For a single relaxation process the following relationship holds (Herzfeld and Litovitz, 1959)

$$\alpha/f^2 = \frac{A\tau}{1 + (2\pi f\tau)^2} + B \qquad (1)$$

where α, f, and τ are the pressure amplitude absorption coefficient, frequency, and relaxation time, respectively; A and B are constants. Values of A, B, and τ were generally determined by the following procedure. In almost all compounds studied B was found from the experimental data to coincide with the absorption due to the solvent alone. The template technique (Piercy and Subrahmanyam, 1965) was then used to measure the maximum value of the absorption per wavelength, μ_{max}, and thus A was determined from the relation (Eigen and de Maeyer, 1963)

$$\mu_{max} = \frac{Av}{2\pi} \qquad (2)$$

where v is the sound velocity. Having A and B, theoretical curves of α/f^2 *vs.* f were generated with differing values of τ and compared with the experimental data to determine relaxation times. This procedure appeared most reliable for obtaining experimental parameters in this study where values of $(\alpha/f^2) - B$ are small. Using these methods we estimate the accuracy of A, B, and τ as ±15, ±3, and ±10%, respectively.

Experiments were generally conducted in the neutral pH

TABLE I: Concentration Dependence of Adenosine Relaxation Process at 35.5°.

Compound	Concn (m)	Urea Concn (M)	$10^7 A/m$ (sec/mcm)	$10^{17}B$ (sec²/cm)	τ (nsec)	$10^{-5}v$ (cm/sec)
Adenosine	0.023		$\approx 3.2^a$	16.4	$\approx 2.7^a$	1.53
	0.046		3.8	16.4	2.9	1.53
	0.029	7.0	3.5	16.2	4.2	1.68
	0.086	7.0	3.4	16.2	4.2	1.68

[a] Approximate value only was determined because of the small amplitude at low concentration.

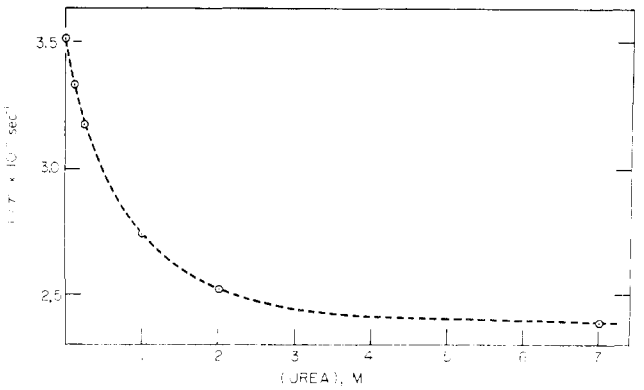


FIGURE 2: Plot of τ^{-1} *vs.* (urea) for adenosine. See Table II for more details.

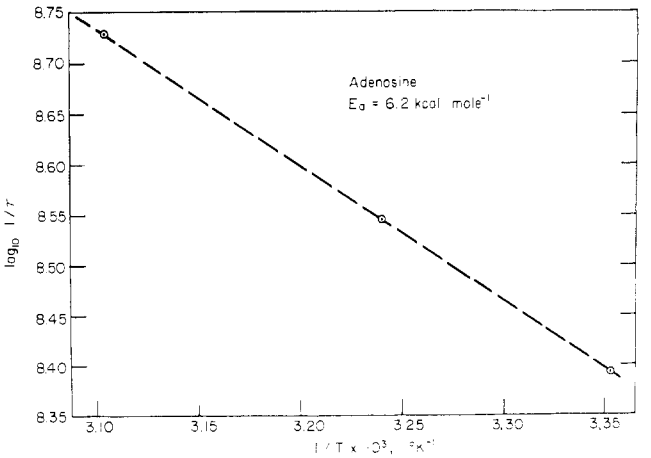


FIGURE 3: Plot of log $(1/\tau)$ *vs.* $1/T$ for adenosine in H_2O. See Table II for more details.

region which is well removed from the pH range of the base ionization reactions. Under these conditions, at the concentrations of nucleosides generally employed (0.01–0.1 M, see below), it is easy to show that contributions to the sound absorbtion of the ionization reactions are completely negligible, even assuming that such reactions have a very large volume change (*e.g.*, 30 cm³/mole; see Eigen and de Maeyer (1963)). Further evidence against contributions from ionization reactions is cited below.

Figure 1 shows a plot of $(\alpha/f^2) - B$ *vs.* f for a solution containing 0.046 M adenosine in H_2O at 35.5°. A single relaxation process is observed with a relaxation time = 2.9 nsec. In order to test for the possibility of base ionization[1] (which is unlikely (see above)) or other bimolecular processes (*e.g.*, base stacking), the concentration dependence of the relaxation time was investigated. However, the concentration dependence of this relaxation process is difficult to investigate in H_2O because of the limited solubility of adenosine. Table I shows that a twofold variation in adenosine concentration gives no significant change in τ or in A/m, as expected for a unimolecular relaxation process. Adenosine is somewhat more soluble in aqueous urea solutions and a relaxation process is also observed in this solvent. Table I also gives the results of a threefold adenosine concentration variation in 7 M urea. The relaxation time τ and A/m are again independent of adenosine concentration. This concentration independence of τ and of A/m in both H_2O and 7 M urea argues strongly that the observed relaxation process is due to a unimolecular reaction. Furthermore, if the relaxation process is in any way due to a concentration dependent process such as base stacking, the relaxation effect should be greatly diminished or abolished in this solvent since 7 M urea disrupts the stacks (E. Jekowsky and P. Schimmel, unpublished data).

The relaxation time is somewhat longer in 7 M urea so that the possibility must be considered that a different process is being observed in H_2O than in 7 M urea. Figure 2 displays $1/\tau$ *vs.* (urea) from 0 to 7 M urea. It is seen that the relaxation time changes continuously from 0 to 2 M urea and then becomes independent of urea concentration. No new relaxation processes appear and the equation (eq 1) for a single process is capable of fitting the data obtained at each urea concentration. This result clearly implies that the same process is being observed in both H_2O and in aqueous urea solutions.

Figure 3 displays the temperature dependence in H_2O of the adenosine relaxation time. The data are plotted as log $(1/\tau)$ *vs.* $(1/T)$; an apparent activation energy of 6.2 kcal mole^{-1} is obtained. In 7 M urea the activation energy is somewhat larger.

Having characterized the adenosine relaxation process, the question naturally arises as to whether or not the same relaxation process is also present in solutions containing the two principal constituents of adenosine, *i.e.*, ribose and adenine. The insolubility (Levine *et al.*, 1963; Robinson and Grant, 1966) of adenine (6-aminopurine) precludes a definitive investigation of this constituent. As an alternative, the considerably more soluble parent compound purine was investigated. It was found that no relaxation effect is pres-

[1] A final piece of evidence against base ionization reactions is that α/f^2 for adenosine in H_2O is the same at pH 5.3 and pH 7.0, at both 25 and 55 MHz.

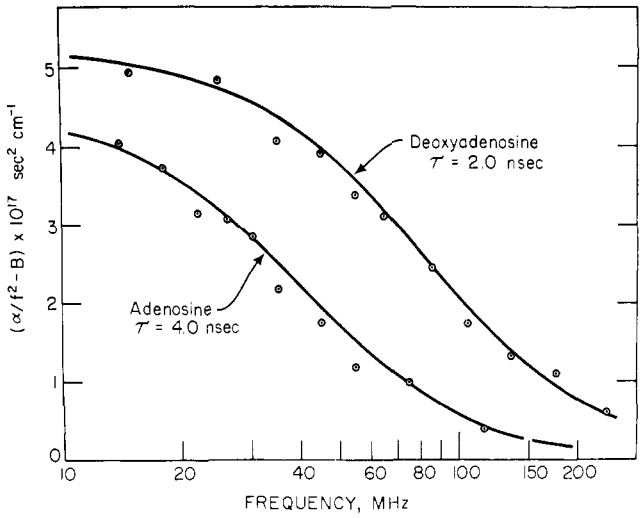


FIGURE 4: Plots of $(\alpha/f^2) - B$ *vs.* f for 0.03 M adenosine at 25° in H_2O and 0.063 M deoxyadenosine at 24° in H_2O. The curves are calculated according to eq 1 using parameters given in Table II.

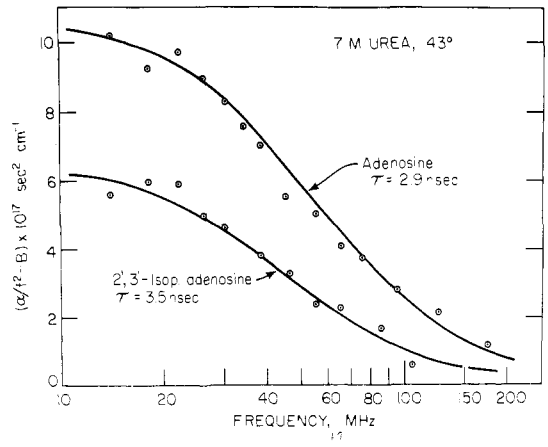


FIGURE 5: Plots of $(\alpha/f^2) - B$ *vs.* f for 2',3'-*O*-isopropylideneadenosine and for adenosine, both in 7 M urea, 43°. The concentration of adenosine is 0.103 *m*; the experiment with isopropylideneadenosine was done at the solubility limit of 0.06 *m* and the data points were then multiplied by 0.1/.06 in order to make a direct comparison with adenosine. The curves are calculated by eq 1 using the parameters given in Table II.

ent in a 0.1 M purine solution at 35.5°. Neither is a relaxation process evident in solutions of 0.1 M ribose at 35.5°. Of course, ribose exists as an equilibrium mixture of five- and six-membered rings and only 15–20% is in the β-furanose form found in nucleosides (Lemieux and Stevens, 1966; Capon, 1969). The addition of phosphate to the 5' position maintains the sugar in the furanose form (Hall, 1964). Solutions of 0.06 M ribose 5-phosphate show evidence of some excess absorption (about 10–20% of H_2O absorption), the amount being much smaller than the amplitude, $A\tau$, of adenosine solutions of comparable concentration. The amount is so small that it is not possible to definitively detect amplitude dispersion. The origin of this very small absorption is not known, but it is present at both pH 4.7 and 6.7. It is conceivably associated in some way with the ionization of the phosphate group (p$K \sim 6$), but more definitive experiments are required.

Since the relaxation process is apparently absent in the base and in the sugar moiety of adenosine, it is of obvious interest to explore the effect of small structural variations in adenosine on the relaxation time. Perhaps the most obvious and important variant of adenosine is 2'-deoxyadenosine. Figure 4 gives plots of $(\alpha/f^2) - B$ *vs.* f for both adenosine (at 25°) and 2'-deoxyadenosine (at 24°). Although both nucleosides show a relaxation effect of comparable amplitude, the relaxation time is a factor of two shorter in deoxyadenosine (τ = 2.0 nsec *vs.* τ = 4.0 nsec for adenosine).

Another adenosine analog of interest is 2',3'-*O*-isopropylideneadenosine (see Chart I). In this nucleoside, the isopropyl group clamps the ribose ring in such a way as to severely restrict the conformational mobility of the ring (see Abraham *et al.*, 1962). Hence, the relaxation process might be expected to disappear if it arises from conformational isomerism of the ribose group. Because of its limited solubility, the isopropylidene derivative can only be studied in 7 M urea at temperatures considerably above the ambient value. Figure 5 gives plots of $(\alpha/f^2) - B$ *vs.* f for adenosine and 2',3'-*O*-isopropylideneadenosine, each in 7 M urea at 43°. It is seen that the relaxation process is also present in solutions of the isopropylidene derivative where τ = 3.5 nsec; under the same conditions τ = 2.9 nsec for adenosine.

Certain other purine nucleosides were also investigated, *i.e.*, guanosine, inosine, and formycin. In all cases a relaxation process was observed which is in the same time range as that of adenosine. The results obtained are summarized in Table II which also includes some of the data obtained with the adenine nucleosides. The data for each nucleoside are not always strictly comparable with each other because experiments were not in every case carried out under the same conditions (owing to differences in solubilities of the nucleosides). It is important to appreciate the fact that it is not feasible to employ nonaqueous solvents (*e.g.*, dimethyl sulfoxide) in which most nucleosides are more soluble. In such solvents relaxation processes corresponding to hydrogen bonding are observed (Hammes and Park, 1968, 1969) which can obscure the effects reported here.

Certain other purine nucleosides not listed in Table II were also investigated. For example, large quantities of 8,-5'-*O*-cyclo-2',3'-*O*-isopropylideneadenosine (see Chart I) were synthesized by the procedure of Ikehara and Kaneko (1968). This is a completely rigid nucleoside of obvious interest for the present investigation, but its insolubility even in 7 M urea precluded a meaningful study. Similarly, 8-bromoadenosine is too insoluble in water and in 7 M urea for a definitive investigation to be carried out. The syn–anti transition should not occur in this nucleoside since the large bromine atom precludes the anti configuration. Finally, an experiment was carried out with the very soluble derivative adenosine 5'-phosphate. Two relaxation processes are observed, one of which appears to be similar to that reported for adenosine alone. It is partly obscured, however, by the second relaxation process which is of considerably greater amplitude. This second process appears to be concentration dependent and preliminary evidence suggests that it is associated with base stacking. Apparently, the negative charge on AMP destabilizes the stacks to the extent that the stacking equilibrium now falls in the high-frequency ultrasonic range. Further investigations of AMP are being carried out.

Two pyrimidine nucleosides were investigated—cytidine and uridine. No relaxation process was observed in solutions of cytidine (see Figure 6). In the case of uridine, faint traces of an

TABLE II: Ultrasonic Parameters for Purine Nucleosides.[a]

Compound	Concn (m)	Urea Concn (M)	Temp (°C)	$10^7 A/m$ (sec/mcm)	$10^{17}B$ (sec²/cm)	τ (nsec)	$10^{-5}v$ (cm/sec)
Adenosine	0.030		25.0	3.7	21.8	4.0	1.50
	0.083		49.0	3.4	12.1	1.9	1.55
	0.046	0.10	35.5	3.8	16.7	3.0	1.54
	0.046	0.25	35.5	3.7	16.6	3.2	1.54
	0.045	1.0	35.5	3.1	16.5	3.7	1.55
	0.045	2.0	35.5	3.0	16.2	4.0	1.56
	0.103	7.0	43.0	3.6	13.8	2.9	1.70
Deoxyadenosine	0.063		24.0	4.2	22.4	2.0	1.51
Formycin	0.030		35.5	5.6	16.4	2.9	1.53
Guanosine	0.033	7.0	35.5	≈2.0[b]	16.2	≈5.7[b]	1.68
2′,3′-*O*-Isopropylideneadenosine	0.060	7.0	43.0	1.8	13.8	3.5	1.69
Inosine	0.10		35.5	0.91	17.4	4.2	1.54

[a] Additional data may be found in Table I. Experiments were done at near neutral pH values. [b] Approximate values only were determined because of guanosine's insolubility.

effect are observable at a concentration of 0.1 M at 25°. This effect becomes more evident at higher concentrations and higher temperatures. Figure 6 gives a plot of α/f^2 *vs.* f for a solution of 0.256 M uridine at 45°, about pH 6. The relaxation effect is clearly below the frequency range accessible to us, and therefore was not further investigated. It should be recognized that the effect with uridine is too small to observe when concentrations (~0.06 M) comparable to those employed with purines are studied.

Discussion

There are three plausible mechanisms which can account for the unimolecular relaxation processes observed in aqueous mononucleoside solutions. These are solvation, puckering, or other equilibria of the ribose ring, and the syn–anti interconversion about the glycosidic bond.

Solvation. Organic molecules in aqueous solution are usually described as being surrounded by a "sphere of hydration" (Klotz, 1958; Kauzmann, 1959; Némethy and Scheraga, 1962; Sinanŏglu and Abdulnur, 1964). The formation and breakdown of this solvating water structure can be responsible for a chemical relaxation process; this is the mechanism referred to as "solvation." There are several examples of this hydration process giving rise to relaxation processes in the ultrasonic frequency range (Hammes and Lewis, 1966; Hammes and Schimmel, 1967; Hammes and Pace, 1968).

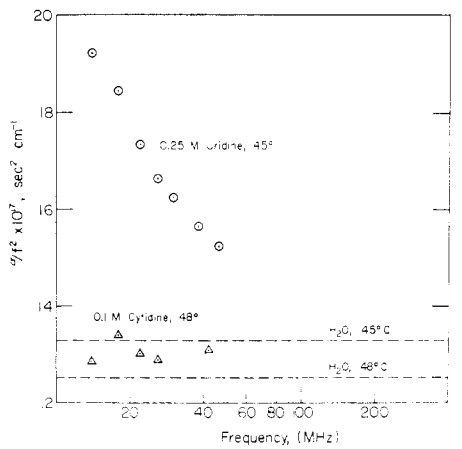


FIGURE 6: Plot of α/f^2 *vs.* f for 0.256 M uridine in H_2O at 45° and for 0.1 M cytidine in H_2O at 48°. The background absorption due to H_2O at both 45 and 48° is indicated by horizontal lines.

An examination of the components of the nucleoside adenosine was undertaken to determine if solvation of the base or ribose moiety could be responsible for the observed relaxation process. Neither aqueous solutions of purine or of ribose showed any detectable relaxation effect, as pointed out above. Furthermore, no relaxation process is present in solutions of cytidine in spite of the fact that it contains the identical ribose moiety as adenosine. These facts argue strongly against solvation of the base or ribose as the mechanism responsible for the relaxation process.

An additional argument against solvation can be advanced by comparing the present results with those of Hammes and Pace (1968) who conducted a detailed study of a relaxation process in aqueous solutions of glycine, diglycine, and triglycine. The authors ascribed this relaxation process to solvation. The relaxation time is identical for glycine, diglycine, and triglycine, and it is independent of temperature (Hammes and Pace, 1968). By contrast, the relaxation effect in the present study is quite sensitive to structural alterations (compare adenosine and deoxyadenosine) and it is quite temperature dependent (*cf.* Figure 3).

Ribose Ring Puckering. It has been known for some time that furanose rings are nonplanar in the solid state and in solution (Jardetsky, 1960, 1961; Lemieux, 1961). The several different puckered conformations have been found in nucleoside and nucleotide crystals (Sundaralingam, 1969), and in fact two distinct puckerings have been observed in the same crystal (Munns *et al.*, 1970; Thewalt *et al.*, 1970; Rahman and Wilson, 1970). These facts indicate that the ribose ring might interconvert between different puckered forms rather easily. In fact, the detailed ribose nmr spectra of many nucleosides and nucleotides (three coupling constants determined) cannot

be satisfactorily accounted for in terms of one of the particular conformations observed in the solid state (Feldman and Agarwal, 1968; Blackburn *et al.*, 1970; Hruska *et al.*, 1970; Hruska, 1971; Grey *et al.*, 1971; Fang *et al.*, 1971). This observation may reflect that these rings are rapidly interconverting between various puckered forms, or that theoretical information concerning the relationship between coupling constants and dihedral angles is incomplete for these compounds.

To test the possibility that a rapid interconversion between puckered forms is responsible for the ultrasonic relaxation process, use was made of the fact that attachment of an isopropylidene group imparts rigidity to the ribose ring (Abraham *et al.*; 1962). Thus, the relaxation effect should not be present in, for example, 2′,3′-*O*-isopropylideneadenosine (see Chart I) if ring puckering is responsible for the relaxation process. The results shown in Figure 5 show that the relaxation process is present in solutions of the isopropylidene derivative and that the time constant is not very different from that of adenosine. This result rules out the possibility that rapid puckering equilibria are responsible for the relaxation process.

Further support for this conclusion comes from the fact that nmr studies suggest the ribose ring of nucleosides is more flexible than the deoxyribose ring (Prestergard and Chan, 1969; Fang *et al.*, 1971). However, the results reported in Figure 4 show that the relaxation process associated with deoxyadenosine is a factor of two shorter than that of adenosine. This is exactly the opposite of what would be expected if the process is due to ring puckering effects with the deoxyribose ring being more rigid.

In addition, no relaxation process is evident in solutions of cytidine in spite of the presence of a β-furanose ring. There is no reason to believe *a priori* that ring puckering reactions should be substantially different or suppressed in cytidine as compared with purine nucleosides.

Finally, other equilibria of the ribose ring that must be considered are the trans–gauche isomerizations of the C'_4–C'_5 bond. This possibility is ruled out by the fact that no relaxation process is observed with cytidine, where the trans–gauche equilibrium should also be free to occur (Wilson and Rahman, 1971).

Syn–Anti Transition. The orientation of the base with respect to the pendant ribose group has been the subject of numerous investigations. Donahue and Trueblood (1960) first pointed out the two possible orientations of the ribose group about the glycosidic bond. These are designated syn and anti, and are crudely illustrated for adenosine in Chart I. X-Ray crystallographic investigations have shown that most β-purine nucleosides and virtually all β-pyrimidines (the exception is 4-thiouridine (Saenger and Scheit, 1970)) are anti in the solid state (Sundaralingam, 1969), although an increasing number of *syn*-purine structures have recently been found (Rao and Sundaralingam, 1970; Travale and Sobell, 1970). In fact, crystals of adenosine 3′,5′-cyclic phosphate possesses two molecules per asymmetric unit, one of which is syn and the other anti (Watenpaugh *et al.*, 1968). These facts suggest that the barrier between syn and anti forms may not be large, especially for purines, and that both syn and anti forms may exist in solution.

This conclusion is supported by some additional lines of evidence. Haschemeyer and Rich (1967) showed that, depending on the sugar puckering, both syn and anti rotamers were sterically possible for purine nucleosides. This conclusion has been confirmed by the more refined calculations of Wilson and Rahman (1971) which suggest that in some structures the syn and anti isomers may be nearly isoenergetic. In addition Miles *et al.* (1971) have conducted a detailed circular dichroism study of guanosine nucleosides, the results of which have been interpreted as demonstrations of the existence of both syn and anti rotamers of these nucleosides in solution.

On the basis of the above considerations, *it is very probable that the relaxation process observed in the present study is due to the syn–anti transition.* Interconversions between rotational isomers in other molecules occur in this time range (Heasell and Lamb, 1956; de Groot and Lamb, 1957). Other possible mechanisms for the observed unimolecular process—solvation and ribose puckering—have been clearly ruled out by experiments cited above.

Relaxation processes were observed in all purine nucleoside solutions investigated. Haschemeyer and Rich (1967), Tinoco *et al.* (1968), Lakshminarayanan and Sasiskharan (1970), and Wilson and Rahman (1971) have all concluded that both syn and anti conformations are sterically feasible for the purine nucleosides. This means that conditions are favorable for observing a relaxation process. For all of the purines investigated, the relaxation times are in the range of 2–6 nsec (Table II). This is not surprising, since their structures are very similar. Differences that do occur can easily be rationalized as arising from subtle differences in base and/or ribose geometries.

From Figure 3 it is seen that the adenosine relaxation time is associated with an activation energy of 6.2 kcal mole^{-1}. Since the relaxation time for a unimolecular process is the sum of rate constants for the forward and reverse reactions, it is not necessarily possible to assign this apparent activation energy to a single rate parameter. However, calculations of Wilson and Rahman (1971) suggest that the syn and anti forms of adenosine are roughly isoenergetic so that the observed activation energy is approximately applicable to each rate constant. It is of interest to compare the observed value of 6.2 kcal mole^{-1} with those values computed by Wilson and Rahman (1971). These authors computed barrier heights using atomic coordinates found for the adenosine moiety of various adenosine containing compounds studied by X-ray crystallography. The results obtained are very sensitive to the coordinates used. For the adenosine in adenosine 2′-uridine dinucleotide, a barrier height of 3.5–4 kcal mole^{-1} was computed (Wilson and Rahman, 1971). For the adenosine in adenosine 5′-phosphate, the barrier height was calculated to be 7 kcal mole^{-1} (Wilson and Rahman, 1971; H. R. Wilson, personal communication). The experimental value of 6.2 kcal mole^{-1} is in good agreement with the range of these calculated values. However, it should be pointed out that by using coordinates from other compounds, much higher barrier heights were calculated (Wilson and Rahman, 1971; H. R. Wilson, personal communication).

The relaxation time at 25° for deoxyadenosine is a factor of two shorter than that for adenosine. This could well be due to the fact that the 2′-hydroxyl can interact sterically with N_3 when rotating between syn and anti if the ribose puckering is C-2′ endo (Haschemeyer and Rich, 1967). On the other hand, it could merely reflect minor differences in atomic coordinates of the two nucleosides which render a more facile rotation for deoxyadenosine.

Figure 2 shows that the adenosine relaxation process is slowed down by about 30% in 7 M aqueous urea. This could result from an effect of the urea on the ribose puckering, and thereby affect the syn–anti interconversion, or it could arise from the increased viscosity of urea solutions over that of water (Kawahara and Tanford, 1966). This increased viscosity

may cause a greater hinderance to rotation. The fact that the relaxation time is not greatly changed by urea has an interesting implication. At the concentrations of adenosine employed here, the nucleoside is known to undergo in water some dimerization *via* stacking (Broom *et al.*, 1967; Solie and Schellman, 1968). The observation that only one relaxation effect is present suggests that both stacked and unstacked bases may behave very similarly. This is confirmed by the fact that urea dissociates stacks (E. Jekowsky and P. Schimmel, unpublished data) and yet causes no large alteration in the relaxation time beyond what might be expected from the effects discussed above. These results are not surprising when considered in the light of models for nucleoside stacks which propose that the pendant ribose groups in a dimer hang off the base moieties in opposite directions (Ts'o *et al.*, 1969). Thus, hinderance to rotation is no greater in the stacked than in the unstacked molecules.

The reason that no relaxation process is observed in solutions of cytidine and only a trace of an effect is evident in uridine solutions (see above) is possibly due to the fact that the preferred conformation of pyrimidines is anti according to several analyses (Haschemeyer and Rich, 1967; Tinoco *et al.*, 1968; Lakshminarayanan and Sasisekharan, 1970). Steric conflicts between the ribose group and O-2 of the pyrimidine ring are responsible for suppressing the syn conformation. If there is a substantial (*e.g.*, tenfold) preponderance of the anti form, the equilibrium between syn and anti may not be sufficiently perturbed by the ultrasonic wave to give rise to an observable relaxation amplitude. In addition, the barrier height between syn and anti may be larger for pyrimidines (H. R. Wilson, personal communication) so that the rate of the transition could be below the frequency range explored here. It should be pointed out, however, that Hart and Davis (1971) and Schweizer *et al.* (1971) have obtained evidence for the existence of both syn and anti forms of pyrimidines in solution.

The effect observed with uridine at 45° at relatively high concentrations (0.256 M) could well be due to the syn–anti transition. The effect appears to be temperature sensitive, but it is too slow to characterize even at 45°. It is almost certainly not due to aggregation (stacking) because uridine has little propensity to self-associate (Solie and Schellman, 1968); in addition, the effect is not abolished by the addition of ~5 M urea.

The amplitude (A/m) of the observed relaxation processes depends upon both the volume change and the enthalpy change associated with the reaction, in addition to the reaction's equilibrium position (Eigen and de Maeyer, 1963). All of these factors are unknown, so that an assessment of any one of them from the data is impossible. Certainly the differences in amplitudes between various nucleosides (see Table II) can arise from differences in any or all of these factors. In aqueous solution, the contribution to the amplitude from the volume change often dominates over that from the enthalpy change (at 4° the enthalpy contribution to the amplitude vanishes (Eigen and de Maeyer, 1963)). Assuming no contribution from the enthalpy term, a minimum ΔV of about ± 3.5 cm^3/mole is estimated for adenosine at 36°. On the other hand, if $\Delta V = 0$, a minimum ΔH of ± 10 kcal mole^{-1} is estimated.

If the enthalpy change of the syn–anti conversion for adenosine is in fact small, as calculations suggest (Wilson and Rahman, 1971), then it is not easy to envision a volume change as large as 3.5 cm^3/mole for this process. Of course, changes in local solvent structure could be responsible for such a volume change. On the other hand, even though the results discussed above are certainly most consistent with the syn–anti transition as the mechanism responsible for the relaxation processes observed, it is conceivable that another unknown mechanism is involved.

Acknowledgment

P. R. S. is grateful to Professor G. G. Hammes for drawings of his ultrasonic cells.

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A New Fluorescent Probe for Protein and Nucleoprotein Conformation. Binding of 7-(*p*-Methoxybenzylamino)-4-nitrobenzoxadiazole to Bovine Trypsinogen and Bacterial Ribosomes*

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ABSTRACT: A new fluorescent compound, whose fluorescence is strongly dependent on the polarity of the solvent, is described as a probe of hydrophobic areas on proteins and nucleoprotein particles. The synthesis and fluorescence of 7-(*p*-methoxybenzylamino)-4-nitrobenz-2-oxa-1,3-diazole (MBD) are described. MBD belongs to a class of compounds which are strongly fluorescent in solvents of low polarity and essentially nonfluorescent in water. When MBD is bound to a protein molecule, the fluorescence is that of the fluorophore in a low polarity solvent, suggesting that MBD is bound to a hydrophobic area on the macromolecule. To test the usefulness, specificity, and sensitivity of this new fluorescent probe, MBD was applied to bacterial ribosomes and to bovine trypsinogen and trypsin. MBD binds tightly to 50S and 70S ribosomal subunits but not to the 30S subunit. A dissociation constant (K_f) of 5–9 μM^{-1} for the 50S and 70S subunits was measured. Binding of MBD to ribosomes is interpreted in terms of a unique hydrophobic pocket created between protein molecules as a result of protein–nucleic acid interaction. MBD differentially binds the zymogen, trypsinogen, and the enzyme, trypsin. The K_f for binding to trypsinogen at pH 5.0 is 50 μM^{-1}, but only $\sim$0.3 mM for trypsin. The decrease in binding of the fluorophore has been followed during the activation of trypsinogen to trypsin and found to follow the kinetics of autoactivation. Changes in binding that occur during this transformation of zymogen to enzyme are described and interpreted in terms of a configurational difference between the inactive and active forms of the protein.

While in recent years X-ray crystallography has provided the structure of several enzyme molecules in the crystalline state, our knowledge of the dynamic aspects of the conformation of proteins in solution has depended on less direct experimental approaches. The spectral properties, ionization states, and chemical reactivity of amino acid side chains have long been used as valuable indicators of side-chain interactions, but often these methods are too limited in sensitivity

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