Kinetics of Hydrogen–Deuterium Exchange in Adenosine 5′-Monophosphate, Adenosine 3′:5′-Monophosphate, and Poly(riboadenylic acid) Determined by Laser-Raman Spectroscopy†

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ABSTRACT: Pseudo-first-order rate constants governing the deuterium exchange of 8-CH groups in adenosine 5′-monophosphate, adenosine 3′:5′-monophosphate, and poly(riboadenylic acid) (poly(rA)) were determined as a function of temperature in the range 20–90°C by means of laser-Raman spectroscopy. For 5′-rAMP, the logarithm of the rate constant exhibits a strictly linear dependence on reciprocal temperature, i.e., $k_\psi = Ae^{-E_a/RT}$, with $A = 2.3 \times 10^{14}$ hr^{-1} and $E_a = 24.2 \pm 0.6$ kcal/mol. For cAMP, above 50°C, k_ψ is nearly identical in magnitude and temperature dependence to that of 5′-rAMP. However, below 50°C, isotope exchange in cAMP is much more rapid than in 5′-rAMP, characterized by a lower activation energy (17.7 kcal/mol) and frequency factor (9.6×10^9 hr^{-1}). Exchange in poly(rA) is considerably slower than in 5′-rAMP at all temperatures, but like cAMP the ln k vs. $1/T$ plot may be divided into high temperature and low temperature domains, each characterized by different Arrhenius parameters. Above 60°C, poly(rA) gives $E_a = 22.0$ kcal/mol and $A = 3.2 \times 10^{12}$ hr^{-1}, while below 60°C, $E_a = 27.7$ kcal/mol and $A = 1.8 \times 10^{16}$ hr^{-1}. Thus, increasing the temperature above 60°C does not diminish the retardation of exchange in poly(rA) vis-a-vis 5′-rAMP. These results indicate that the distribution of electrons in the adenine ring of cAMP is altered by lowering the temperature below 50°C, although no similar perturbation occurs for 5′-rAMP. Retardation of exchange in poly(rA) is most probably due to base stacking at lower temperatures and to steric hindrance from the ribopolymer backbone at higher temperatures. We also report the spectral effects of deuterium exchange on the vibrational Raman frequencies of 5′-rAMP, cAMP, and poly(rA) and suggest a number of new assignments for the 5′ and cyclic ribosyl phosphate groups.

Isotopic hydrogen exchange at the C-8 position of purines was first accomplished by Eidinoff and Knoll (1953) using a platinum catalyst. Exchange in the absence of catalyst was later detected in purine by proton magnetic resonance (^{1}H NMR) spectroscopy of D_2O solutions (Schweizer et al., 1964; Bullock and Jardetzky, 1964). More recently, the lability of the 8-CH group in purine nucleosides, nucleotides, and nucleic acids has been demonstrated by the incorporation of tritium from T_2O solution (Osterman et al., 1966; Shelton and Clark, 1967; Maslova et al., 1969a) and by ^{1}H NMR, infrared, and Raman spectroscopy of D_2O solutions (McDonald et al., 1965; Fritzsche, 1967; Medeiros and Thomas, 1971; Livramento and Thomas, 1974). The exchange reaction at C-8 is typically several orders of magnitude slower than the exchange of amino-group protons in purine nucleotides (McConnell and Seawell, 1972) and is thus rather easily monitored by spectroscopic methods. The slow exchange is also of considerable practical value, since DNA or RNA tritiated in such manner can be profitably employed in hybridization studies (Searcy, 1968; Doppler-Bernardi and Felsenfeld, 1969) and in conformational analyses (Gamble and Schimmel, 1974).

Several workers have relied upon tritium exchange and scintillation counting techniques to determine the kinetics of 8-CH exchange in various purines. Maslova et al. (1969a,b) reported a fourfold retardation of the exchange rate for adenine residues in single-stranded poly(rA)[1] and an 80-fold retardation for adenine residues in double-stranded poly(rA$^+$)·poly(rA$^+$), each compared with adenosine 5′-monophosphate. Retardation was attributed in poly(rA) to base-stacking interactions and in poly(rA$^+$)·poly(rA$^+$) to base-pairing interactions, on the assumption that such secondary structures will localize electron density in the 8-CH bond. In an earlier study, Osterman et al. (1966) suggested that base paired secondary structure of T2-DNA had no appreciable effect on the overall rate of 8-CH exchange of adenine and guanine nucleotides, yet the presence of secondary and tertiary structures in tRNA has indeed been shown to retard the rate of exchange of its purine residues (Gamble and Schimmel, 1974), thus supporting the poly(rA) studies.

Elvidge et al. (1971) reported for adenosine a pseudo-first-order rate constant for 8-CH exchange (k_ψ) twice as large as that of purine or adenine, suggesting an electron withdrawal role for the ribofuranosyl group. The authors also estimated Arrhenius parameters for k_ψ and cited evidence for exchange of the 2-CH group in adenosine, though with a 10^3-fold lower rate than that governing exchange of 8-CH. In a more comprehensive study, Tomasz et al. (1972) determined k_ψ for numerous adenosine and guanosine derivatives, noting a pH dependence of k_ψ only in the latter compounds. Mechanisms which were proposed to explain these results suggested an important role for the

† From the Department of Chemistry, Southeastern Massachusetts University, North Dartmouth, Massachusetts 02747. *Received March 17, 1975.* Part XIV in the series Raman Spectral Studies of Nucleic Acids. Part XIII is Livramento and Thomas, 1974. This work was supported by National Science Foundation Grant GB41382 and Public Health Service Grant AI-11855-01. The support of the National Science Foundation and the National Institute of Allergy and Infectious Diseases is gratefully acknowledged.

[1] Abbreviation used is poly(rA), poly(riboadenylic acid).

guanine zwitterion. Further investigations of 8-CH exchange in purine nucleosides (Elvidge et al., 1973a–c, 1974) have resulted in the proposal of a somewhat different mechanism for exchange in guanines but have confirmed earlier conclusions regarding the prevailing mechanism for exchange in adenines. Thus there appears to be a consensus that adenines exchange by way of an ylide intermediate formed after protonation of the N-7 ring position (Tomasz et al., 1972). The evidence is also convincing that base-stacking and base-pairing interactions perturb the rate of 8-CH exchange in polynucleotides and nucleic acids.

Recently we have shown that the exchange kinetics can be more accurately and easily determined by laser-Raman spectroscopy of D_2O solutions (Livramento and Thomas, 1974). A single Raman spectrum permits the 8-CH and 8-CD forms of adenine to be distinguished from one another while at the same time their relative concentrations are quantitatively determined by the intensities of the Raman scattering. The same is true for guanine derivatives (McMahon and Thomas, unpublished results). Compared with tritium-labeling techniques, the Raman method offers advantages of simplicity, speed, and versatility, as well as improved precision in the determination of rate constants and their temperature dependence.

In this paper we present a detailed comparative study of the kinetics of hydrogen–deuterium exchange at the C-8 positions of adenosine 5′-monophosphate (5′-rAMP), adenosine 3′:5′-monophosphate or cyclic AMP (cAMP), and poly(riboadenylic acid) (poly(rA)). The rate data were obtained over a wide temperature range (20–90°C) and provide Arrhenius parameters for each compound. Since the present method depends upon the use of Raman lines of the nucleotide phosphate groups as internal standards, reliable assignments and intensity measurements were required. Therefore, we have included in this study a series of Raman spectra of 5′-rAMP and cAMP to evaluate the effects of pH and pD changes on the phosphate-group frequencies and intensities apart from 8-CH exchange. These spectra also reveal the adenine ring vibrations that are sensitive to deuteration of cAMP and complement previously published results on 5′-rAMP (Lord and Thomas, 1967) and poly(rA) (Prescott et al., 1974).

The present results are of interest for several reasons. First, it is anticipated that 8-CH exchange rates will depend upon details of molecular structure and conformation in the immediate vicinity of the exchangeable groups (Englander, 1963). Therefore comparative rate determinations on 5′-rAMP, cAMP, and poly(rA) provide a basis for evaluating the effects of base-stacked secondary structure and differing ribosyl phosphate structures on the lability and accessibility of the 8-CH bond. Second, normal coordinate calculations on the adenine residue (Tsuboi et al., 1973) become simplified when vibrational frequencies of isotopically substituted derivatives such as 8-CD forms are available. Third, the Raman spectra of D_2O solutions of nucleic acids are a valuable source of information on temperature-dependent secondary structures of nucleic acids (Lafleur et al., 1972; Hartman et al., 1973; Chen and Thomas, 1974). However, utilization of the Raman data for such purposes requires that the spectral changes generated by exchange of labile hydrogen atoms be understood beforehand. Finally, the purines serve as good model systems to evaluate whether a significant isotope effect (i.e., hydrogen–deuterium vs. hydrogen–tritium exchange) occurs in the exchange of -CH pseudoacids. Comparison of the present results with the results of tritiation studies may shed further light on this question.

Experimental Procedures

Sample Handling. 5′-rAMP and cAMP (sodium salts; Sigma) were dissolved in H_2O or D_2O (99.8% deuterium; Aldrich) and the pH or pD of solution was adjusted as required by addition of NaOH and HCl or NaOD and DCl (Aldrich). Poly(rA) (potassium salt; Miles) was dissolved at pD 7.0 to 40 $\mu g/\mu l$ in D_2O. At pD 7.0 the adenine residues of poly(rA) are not protonated and hence formation of the complex poly(rA^+)·poly(rA^+) is not likely. That poly(rA) was indeed in the neutral, single-stranded form is confirmed by the Raman spectra which reveal all of the frequencies expected of poly(rA) (Prescott et al., 1974) and none of the frequencies characteristic of either the protonated adenine residue or the double-helical complex (cf. Figure 3 with Raman spectra of protonated adenines (Lord and Thomas, 1967) and poly(rA^+)·poly(rA^+) (Yu, 1969; Thomas and Hartman, 1973)).

D_2O solutions were prepared and handled in a cold room (4°C) to minimize the slow exchange of 8-CH groups prior to incubation. Identical rate data were obtained on samples previously lyophilized from D_2O, indicating that no significant advantage resulted from such attempts to remove residual H_2O of crystallization from the desiccated solids. Assuming two molecules of H_2O per nucleotide residue in the commercial samples, deuterium content of the stock solutions was in no case less than 97.5 mol %.

Aliquots (20 μl) of the D_2O stock solutions were introduced into Raman cells (Kimax 34507 capillaries) and sealed. Cells were then placed in a constant temperature bath and incubated at the specified temperature (to ±0.5°C) to promote exchange. Cells were removed periodically from the bath and transferred immediately to a 0°C thermostat in the Raman spectrometer for recording of spectra. In a typical run, e.g., to determine k_ψ (80°C), of 5′-rAMP, 20 cells were incubated at 80°C and one cell was removed every half-hour over a period of 10 hr to provide a series of data points in the first-order rate analysis (plot of ln concentration vs. time). Additional cells were examined after sufficient time to ensure complete exchange, in this case 30 hr. Two or more spectral scans were made on each cell. Similar series of spectra were recorded on samples incubated at other temperatures in the 20–90°C interval to provide sufficient data to evaluate the Arrhenius parameters for 5′-rAMP, cAMP, and poly(rA).

Instrumentation. Raman spectra were recorded on a Spex Ramalog spectrometer using 488.0-nm excitation from a Coherent CR-2 argon-ion laser. The radiant power at the sample was in the range 200–300 mW. Further details of Raman instrumentation are given elsewhere (Thomas and Barylski, 1970; Medeiros and Thomas, 1971).

Total nucleotide concentrations were determined precisely by diluting aliquots of the stock solutions 1:1000 in phosphate pH 7.5 buffer and recording uv absorption spectra on a Beckman Model DK-2A spectrometer.

pH and pD (=pH + 0.4) measurements were made on a Beckman Expandomatic SS-2 meter with a Fisher microelectrode.

Data Handling. Concentrations of 8-CH and 8-CD forms of adenine are proportional to the intensities of 1485- and 1462-cm^{-1} lines, respectively, in the Raman spectrum (Livramento and Thomas, 1974). The rate of exchange may therefore be determined by either the rate of intensity decay

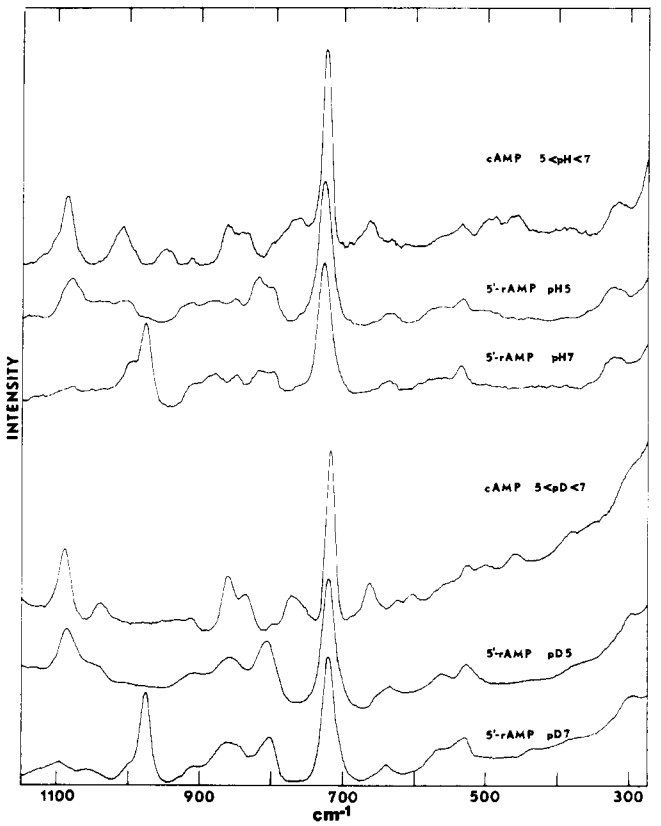


FIGURE 1: Raman spectra in the region 300–1100 cm^{-1} of H_2O and D_2O solutions of 5′-rAMP and cAMP. All spectra are those of the 8-CH form of the adenine ring. Conditions: concentration c, 0.25 M, excitation wavelength λ, 488.0 nm, scan rate r, 50 cm^{-1}/min, spectral slit width $\Delta\sigma$, 10 cm^{-1}, rise time t, 1 sec. For Raman spectra above 1100 cm^{-1} and for spectral effects of 8-CH deuterations see Figures 2 and 3.

at 1485 cm^{-1} or the rate of intensity increase at 1462 cm^{-1}. These two independent methods of calculating k_ψ provide results which are in close agreement (±10%) with one another, but the former is considered to be more accurate (subject to uncertainties of ±5% or less) for the following reasons. First, the CH deformations of ribose in 5′-rAMP and poly(rA) give rise to very weak Raman scattering near 1460 cm^{-1} which complicates the intensity measurements at this frequency, particularly during the early stages of exchange. Second, after prolonged incubation, beyond the time required to "complete" exchange at C-8, the 1462-cm^{-1} line appears to increase marginally in intensity. This is attributed to the onset of exchange at the C-2 position of adenine, governed by a much lower rate constant (Elvidge et al., 1971). Consequently we place greater reliability on rate constants determined from the Raman data at 1485 cm^{-1}.

Intense Raman lines of the phosphate groups, which are unaffected by deuterium substitutions in the adenine ring (or ribose moiety), are used as internal standards for quantifying the 1485-cm^{-1} intensity in terms of molecular concentration. These lines are centered at approximately 980 cm^{-1} (5′-rAMP), 1090 cm^{-1} (cAMP), and 1095 cm^{-1} (poly(rA)). Furthermore, since all spectra are recorded at the same temperature (0°C) and pH (7.0), the phosphate line intensities are not subject to changes in effective phosphate ion concentration in a given run. It may be added that the phosphate line intensities will not vary significantly even should variations of temperature or pH (within a few tenths of a pH unit) occur (Lord and Thomas, 1967; Thomas and Hartman, 1973). The 980-cm^{-1} line is assigned to the symmetric PO_3^{2-} stretching vibration of 5′-rAMP and the 1090–1095-cm^{-1} lines to the symmetric PO_2^- stretching vibration of cAMP and poly(rA).

Raman intensities were determined by measuring the peak height above a base line tangent to the wings of the peak in question. This method provided results which were within experimental error of those obtained by an alternative but more time consuming band area method.

Accordingly, the concentrations at time t, $[C_H{}^t]$ and $[C_D{}^t]$, of nonexchanged and exchanged residues, respectively, are

$$[C_H{}^t] = I_{1485}{}^t - I_{1485}{}^\infty = \gamma(I_{1465}{}^\infty - I_{1465}{}^t)$$

$$[C_D{}^t] = I_{1485}{}^0 - I_{1485}{}^t = \gamma(I_{1465}{}^t - I_{1465}{}^0)$$

and

$$[C_H{}^0] = [C_D{}^\infty] = I_{1485}{}^0 - I_{1485}{}^\infty = \gamma(I_{1465}{}^\infty - I_{1465}{}^0)$$

where $I_\sigma{}^t$ is the Raman intensity at frequency σ after exchange time t, normalized to the intensity of the internal standard; $[C_H{}^0]$ is determined from uv absorbance measurement; $[C_H{}^\infty] = [C_D{}^0] = 0$ by assumption; and γ is the ratio of the intrinsic intensities of the 1485- and 1462-cm^{-1} lines. For pseudo-first-order kinetics

$$\ln \frac{[C_H{}^0]}{[C_H]} = \ln \frac{I_{1485}{}^0 - I_{1485}{}^\infty}{I_{1485}{}^t - I_{1485}{}^\infty} = k_\psi t \qquad (1)$$

or

$$\ln \frac{[C_H{}^0]}{[C_H]} = \ln \frac{I_{1465}{}^\infty - I_{1465}{}^0}{I_{1465}{}^\infty - I_{1465}{}^t} = k_\psi t \qquad (2)$$

and a plot of $\ln [C_H{}^0]/[C_H]$ vs. t should exhibit slope k_ψ. As stated earlier, eq 1 provides the more accurate results.

Results and Discussion

1. Raman Spectra of cAMP (8-CH Forms). In this section the term deuteration signifies the replacement of amino and hydroxy group hydrogens by deuterium and not the replacement of the C-8 hydrogen by deuterium.

Raman spectra of adenosine and 5′-rAMP have been discussed previously (Lord and Thomas, 1967), as have spectra of ADP and ATP (Rimai et al., 1969). Spectra of cAMP and 5′-rAMP are very similar, since the strongest Raman scattering in these compounds originates mainly from vibrations of the adenine ring. Major differences are found only in those spectral ranges where the ribose and phosphate residues contribute appreciably. These are at 300–1100 cm^{-1} (skeletal stretching of ribose and P–O stretching of phosphate groups), ca. 1460 cm^{-1} (C–H deformations of ribose), and 2800–3000 cm^{-1} (C–H stretching of ribose).

Figure 1 compares the Raman spectra of cAMP and 5′-rAMP in the region 300–1100 cm^{-1} for both H_2O and D_2O solutions. The strongest line in each spectrum (ca. 725 cm^{-1}) is assigned to the adenine ring (Lord and Thomas, 1967), but many of the weaker lines are seen to be due to the ribose and phosphate residues since their frequencies and intensities are sensitive to one or more of the following factors: deuteration of OH groups of ribose (i.e., change of solvent from H_2O to D_2O), cyclization of the phosphate group, and change of pH or pD in the range 5.0–7.0.

Figure 1 confirms a number of earlier observations and assignments and suggests additional new assignments. For

H_2O (D_2O) solutions, the lines near 325 (300), 385 (375), 535 (530), 570 (560), and 915 (915) cm^{-1} are not all present in spectra of other purine or pyrimidine nucleotides and therefore, like the 725 (720)-cm^{-1} line, are assigned to the neutral adenine ring. The small deuteration shifts indicate that these frequencies originate mainly from ring skeletal motions. The line at 980 cm^{-1} in 5′-rAMP at pH or pD 7 is due to symmetric P–O stretching of the PO_3^{2-} group. At pH or pD 5, when 90% of the phosphate groups of 5′-rAMP attach a proton, the 980-cm^{-1} line is replaced by a line near 1085 cm^{-1} which is due to symmetric P–O stretching of the PO_2^- group. A line near this frequency is also observed in cyclic and polynucleotides, both of which have the PO_2^- group (Thomas and Hartman, 1973). Degenerate stretching of the PO_3^{2-} group and antisymmetric stretching of the PO_2^- group are not intense in the Raman effect but may contribute to the very weak Raman scattering in the region 1100–1230 cm^{-1} (Shimanouchi et al., 1964). There is no Raman line which can be assigned with confidence to P–O stretching of the phosphomonoester group in 5′-rAMP or in other mononucleotide divalent anions. However, at pH 5 the 5′-rAMP monoanion gives enhanced scattering near 820 cm^{-1} which we assign to symmetric P–O stretching in the 5′C–O–P–O–H network. At pD 5 this frequency occurs at 810 cm^{-1} and the small deuteration shift suggests a nearly pure P–O stretching vibration. A line near 810–820 cm^{-1} also occurs in Raman spectra of polynucleotides (Thomas and Hartman, 1973), oligonucleotides (Prescott et al., 1974), RNA (Thomas, 1970), and 5′-rIMP monoanions (Medeiros and Thomas, 1971) assignable to the O–P–O symmetric stretch. That poly(rA) does not exist in the protonated double-helical form is confirmed by the absence of Raman lines at 824 and 1562 cm^{-1} (Yu, 1969).

It is interesting to note that cAMP gives no prominent Raman line between 775 and 825 cm^{-1}. This result is not surprising, for although the O–P–O diester linkage is present in cAMP, it is well established (Shimanouchi et al., 1964; Thomas and Hartman, 1973) that the O–P–O diester stretching frequency is highly sensitive to the geometry of the 5′C–O–P–O–3′C network. In cAMP the diester group is incorporated into a ring where the bond angles are significantly different from those occurring in the polynucleotide or oligonucleotide structures (Sundaralingam, 1969, 1973; Watenpaugh, et al., 1968). Furthermore, the "group frequency" concept which applies quite well for polynucleotide diester groups may not apply for cyclic nucleotides where mechanical coupling of ring C–C, C–O, and O–P bond stretching motions apparently generates a very different pattern of Raman frequencies.

We assign the 771-, 837-, and 863-cm^{-1} frequencies, all of which exhibit little or no deuteration shift, to the phosphate-ester ring of cAMP. The lines at 458, 495, 610, 634, 663, 685, and 800 cm^{-1}, which also exhibit little or no deuteration effect, originate in either the phosphate ester or ribofuranosyl rings of cAMP. The lines at 950, 1010, and 1115 cm^{-1} are present only in H_2O solutions of cAMP, while the line at 1038 cm^{-1} appears only for the D_2O solution of cAMP. These latter frequencies may be associated with the 2′C–O external group and ribosyl ring stretching. Figure 1 also indicates that the ribose 5′-phosphate residue has few frequencies in common with the cyclic phosphate residue. Where frequencies are coincident, intensities are generally different. We suggest that only the line near 635 cm^{-1} in all spectra has a common origin in 5′-rAMP and

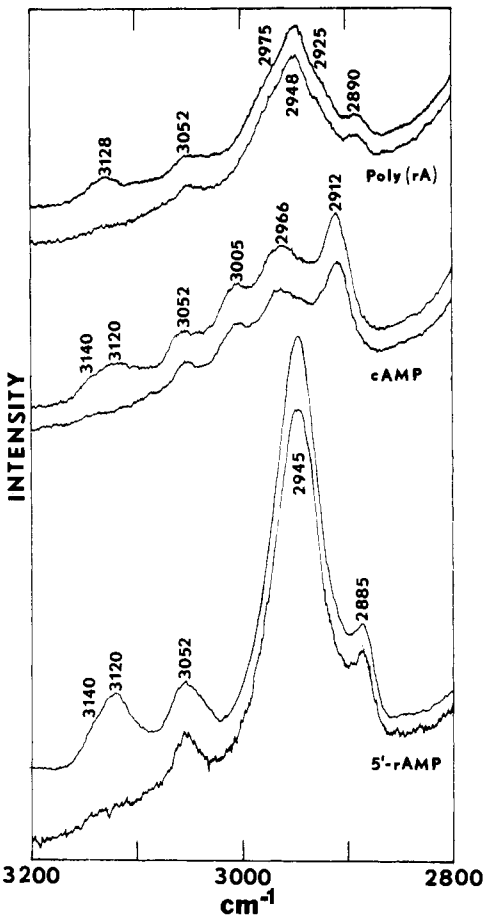


FIGURE 2: Raman spectra in the region 2800–3200 cm^{-1} of D_2O solutions of 5′-rAMP, cAMP, and poly(rA). For each pair of spectra the upper curve corresponds to the 8-CH form and the lower curve to the 8-CD form of the adenine ring. Conditions as in Figure 1.

cAMP, probably in the 2′C-1′–C–O linkages. The line near 915 cm^{-1} in all spectra is removed by 8-CH deuteration and therefore must originate in the adenine ring and not in the ribose phosphate moiety (Livramento and Thomas, 1974; see also Figure 3, below).

In the region 1100–1800 cm^{-1}, spectra of H_2O and D_2O solutions of cAMP are virtually identical with spectra of corresponding solutions of 5′-rAMP published previously (Lord and Thomas, 1967), with the following exception. The weak line at 1460 cm^{-1} in 5′-rAMP, assigned to ribosyl C–H deformations, is absent from spectra of cAMP. The corresponding C–H deformations of cAMP presumably occur at somewhat higher or lower frequencies where they are masked by other Raman scattering from adenine ring vibrations.

Structural differences between 5′-rAMP and cAMP are also revealed in the region of ribosyl C–H stretching vibrations (2800–3000 cm^{-1}), as shown in Figure 2. Thus the C–H stretching frequencies of ribose in 5′-rAMP give rise to Raman lines centered at 2885 and 2945 cm^{-1}. The latter is very intense and broad and no doubt represents the superposition of several closely spaced but poorly resolved Raman frequencies. For cAMP, on the other hand, ribosyl C–H stretching gives well-resolved Raman lines at 2912, 2966, and 3005 cm^{-1}. The ribosyl C–H stretching frequencies of poly(rA) more closely resemble those of 5′-rAMP than cAMP, as expected (Figure 2).

Well above 3000 cm^{-1} are found the aromatic C–H stretching frequencies of the 2-CH and 8-CH groups of ad-

Table I: Raman Frequencies and Assignments of Adenine Nucleotides.[a]

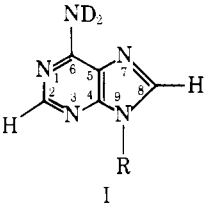
cAMP		5′-rAMP				
5 < pH < 7	5 < pD < 7	pH 5	pH 7	pD 5	pD 7	Assignments[b]
320 (0B)	300 (0B)	325 (1B)	325 (1B)	300 (0B)	300 (0B)	A ring
383 (0)	390 (0)	385 (0)	385 (0)	385 (0)	385 (0)	A ring
458 (0)	458 (0)					RP
495 (0)	500 (0)	500 (0)	495 (0)			RP
530 (0)	530 (0)	535 (0)	535 (0)	528 (1)	530 (1)	A ring
560 (0)	560 (0)	570 (0)	570 (0)	562 (0)	560 (0)	A ring
610 (0)	605 (0)					RP
634 (0)	625 (0)	637 (0)	637 (0)	635 (0)	635 (0)	R(2′C-1′C-O str)
663 (1)	665 (1)					RP
685 (0)	685 (0)					RP
726 (8)	718 (7)	728 (5)	727 (5)	720 (4)	721 (4)	A ring
771 (1B)	774 (1B)					P ring
800 (0)	800 (0)	800 (S)	800 (S)			RP
		818 (1)	818 (S)	805 (1B)	802 (1B)	P(O–P–O sym str)
837 (1)	835 (1)					P
		852 (0)	850 (1)			RP
863 (1)	861 (2)					P
		885 (1)	885 (1)	860 (1B)	860 (1B)	RP
913 (0)	915 (0)	915 (0)	912 (0)	910 (0)	910 (0)	A ring
950 (1B)	925 (0)					R(C–O str)
			978 (3)		978 (3)	$P(PO_3^{2-}$ sym str)
1010 (2)		1005 (0)	1000 (S)	1000 (0)	1000 (S)	R(C–O str)
1025 (S)	1038 (1)	1045 (0)	1045 (0)	1050 (0B)	1050 (0B)	R(C–O, C–C str)
1086 (3)	1090 (3)	1084 (2)		1085 (2)		$P(PO_2^-$ sym str)
1115 (0)		1135 (0)	1135 (0)			R(C–C str)
1175 (1)	1183 (?)	1177 (0)	1175 (0)	1183 (?)	1183 (?)	A ring
1215 (0)		1220 (1)	1218 (1)			A ring
1250 (1B)		1253 (2)	1251 (2)			A ring
1308 (6)	1305 (7)	1308 (6)	1308 (6)	1307 (6)	1307 (6)	A ring
1337 (10)	1340 (10)	1337 (10)	1337 (10)	1340 (10)	1340 (10)	A ring
1375 (4)	1381 (3)	1377 (4)	1377 (4)	1383 (3)	1383 (3)	A ring
1425 (0)	1425 (0)	1422 (1)	1422 (1)	1426 (0)	1426 (0)	A ring
		1460 (0)	1460 (0)	1460 (0)	1460 (0)	R(C–H def)
1483 (3)	1485 (4)	1485 (3)	1483 (3)	1485 (4)	1485 (4)	A ring
1508 (3)	1520 (2)	1508 (3)	1508 (3)	1520 (1)	1520 (1)	A ring
1580 (5)	1578 (4)	1579 (5)	1580 (5)	1577 (4)	1577 (4)	A ring
	1622 (1)			1624 (0)	1624 (0)	A ring
2912 (3)	2912 (3)	2885 (2)	2885 (2)	2885 (2)	2885 (2)	R(C–H str)
2966 (2)	2966 (2B)	2945 (8)	2945 (8)	2945 (8B)	2945 (8B)	R(C–H str)
	3005 (2B)					R(C–H str)
	3053 (1B)			3053 (1)	3053 (1)	A(2C–H str)
	3120 (1)			3120 (1)	3120 (1)	A(8C–H str)
	3140 (S)			3140 (S)	3140 (S)	A(overtone 2 × 1577)

[a] Frequencies in cm^{-1} are accurate to ±2 cm^{-1} for strong lines and to ±4 cm^{-1} for weak or broad lines. Numbers in parentheses give the relative intensities in each spectrum on a 0 to 10 scale. Abbreviations: B broad, S shoulder. In H_2O solutions, frequencies above 3000 cm^{-1} are obscured by the solvent. [b] Abbreviations: A, adenine; RP, ribose phosphate moiety; R, ribose group; P, phosphate group; sym, symmetric; str, stretching; def, deformation.

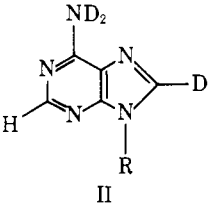
enine. In 5′-rAMP and cAMP these frequencies occur at 3052 and 3120 cm^{-1}. The assignments are unambiguous, since the 3120 cm^{-1} line disappears upon C-8 deuteration, as shown in Figure 2. For poly(rA), the corresponding Raman assignments are at 3052 and 3128 cm^{-1}, again supported by the fact that C-8 deuteration eliminates only the 3128-cm^{-1} component. The slightly higher value of the 8-CH stretching frequency in poly(rA) as compared with 5′-rAMP and cAMP (3128 vs. 3120 cm^{-1}) could result either from base stacking in the polymer, or from Fermi resonance involving the overtone of the adenine ring fundamental at 1577 cm^{-1}, or from both of these factors. We are unable to locate the 8C–D stretching frequency, expected near 2200 cm^{-1} because of the strong Raman scattering of D_2O which obscures this spectral region.

All of the Raman assignments discussed in this section are summarized in Table I. Effects of C-8 deuteration on the Raman spectra of adenine derivatives are discussed in detail in the subsequent section.

2. Spectral Effects Resulting from Deuterium Exchange of the Adenine 8-CH Group in 5′-rAMP, cAMP, and Poly-(rA). In this section we review the spectral effects produced by deuteration of the adenine 8-CH group in 5′-rAMP and poly(rA) (Livramento and Thomas, 1974) and report the results of 8-CH deuteration of cAMP. Figure 3 compares the Raman spectra in the region 300–1800 cm^{-1} of D_2O solutions of 5′-rAMP, cAMP, and poly(rA), both before and after C-8 deuteration of each compound. The solid and broken curves of Figure 3 are spectra corresponding respectively to the adenine ring structures I and II, where R is the ap-



I



II

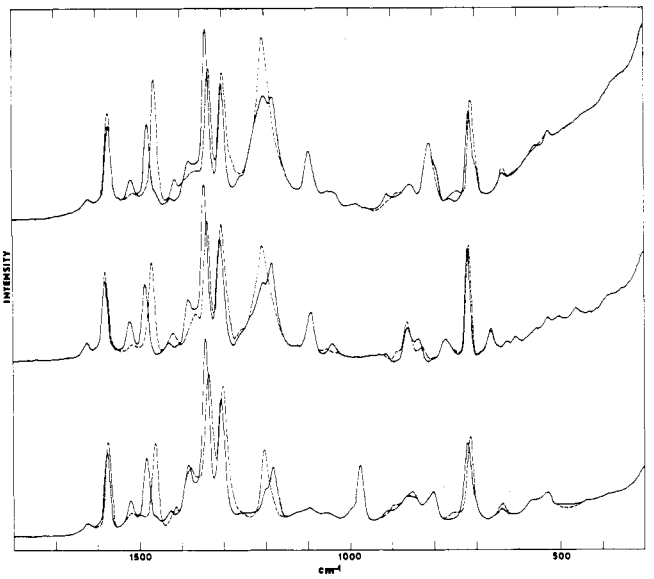


FIGURE 3: Raman spectra of D_2O solutions of 5′-rAMP (lower), cAMP (middle), and poly(rA) (upper). (—) 8 C-H form of adenine ring, (- - -) 8C-D form of adenine ring. Conditions as in Figure 1, except poly(rA) concentration is 40 μg/μl (~0.10 M in adenine).

propriate glycosidic substituent. All hydroxyl groups of ribose are in the OD form. Raman data for the region 2800–3200 cm^{-1} were presented in Figure 2.

Figure 3 indicates that the Raman lines near 1520, 1485, 1425, 1380, 1340, 1305, 1183, 915, and 718 cm^{-1}, common to each 8-CH derivative, are displaced upon deuteration. Therefore all of these adenine ring frequencies are implicated to some extent with the in-plane or out-of-plane deformations of the 8-CH group. Likewise, the new Raman lines near 1515, 1462, 1410, 1370, 1332, 1300, 1201, 895, 850, 750, and 713 cm^{-1}, common to all 8-CD forms, are implicated with the 8-CD deformations. Figure 2 indicates not surprisingly that the line near 3120 cm^{-1} in each 8-CD derivative is due to a relatively pure 8-CH stretching vibration.

The 8-CH and 8-CD forms of adenine are most easily distinguished in the Raman spectrum by their intense and well-resolved lines at 1485 and 1462 cm^{-1}, respectively. These Raman lines are also well-adapted to precise intensity measurement for purposes of determining the exchange kinetics (Experimental Section, and Livramento and Thomas, 1974).

3. Kinetics of Exchange in 5′-rAMP, cAMP, and Poly(rA). Figure 4 shows that deuterium exchange of 8-CH groups in 5′-rAMP, cAMP, and poly(rA) is indeed a first-order process. Pseudo-first-order rate constants at several temperatures in the range 20–90°C are listed in Table II. The precision and reproducibility of the measurements are demonstrated by the fact that seven independent determinations on 5′-rAMP at 80°C yielded a mean value for k_ψ of 0.218 hr^{-1} with a standard deviation of 0.010 hr^{-1}. Two or more independent determinations yielded the remaining mean values of k_ψ entered in Table II. All results are based on the intensity decay of the 1485-cm^{-1} line as a function of the time of heating at the temperatures specified in Table II. (Approximate k_ψ values determined from the rate of intensity increase at 1462 cm^{-1} are within ±10% in each case. See also Experimental Procedures.)

The data of Table II are plotted in Figure 5 which indicates the following. For 5′-rAMP, the dependence of ln k_ψ on $1/T$ is strictly linear throughout the range 20–80°C. Considering 5′-rAMP as the norm, exchange in cAMP is also normal at temperatures above 50°C. However, below 50°C, exchange in cAMP is more rapid than expected (e.g., at 37°C, nearly a factor of two greater than the exchange rate of 5′-rAMP). This abnormally rapid exchange in cAMP is accurately reproducible. Furthermore Raman spectra of exchanged cAMP reveal no evidence of hydrolysis of ester linkages or other chemical modification of its molecular structure. The present findings therefore must be attributed to either a conformational change in cAMP or to some type of associative interaction at lower temperatures which affects the electron distribution in the vicinity of the C-8 ring position.

On the other hand, Figure 5 shows that the exchange rate of poly(rA) is abnormally slow at all temperatures. Below 60°C, poly(rA) exhibits a pattern of exchange which, if extrapolated to higher temperatures, would approach that of 5′-rAMP and finally become identical with it at approximately 100°C. In this respect our results are similar to those reported by Maslova et al. (1969a,b) who predicted that the exchange rate of poly(rA) would equal that of 5′-rAMP at ca. 100°C when retardation caused by base stacking in the polymer is effectively eliminated. However, such an extrapolation is not justified by the actual data. Our results indicate that at higher temperatures (>60°C) the exchange rate in poly(rA) follows a quite different pattern than is exhibited at the lower temperatures. Instead of ap-

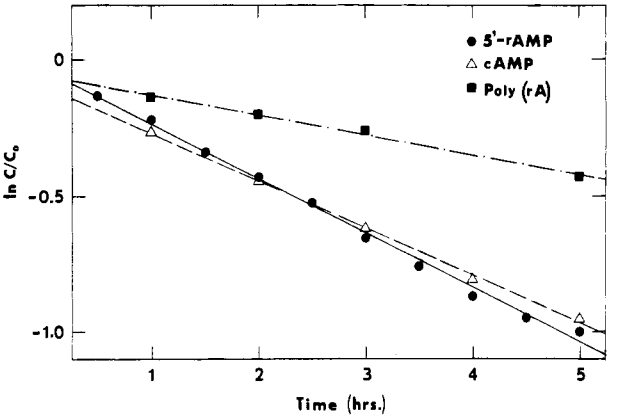


FIGURE 4: Semilogarithmic plots of concentration of nonexchanged adenine vs. time of heating at 80°C. At 5 hr, approximately 67% of the 5′-rAMP residues are exchanged.

Table II: Pseudo-First-Order Rate Constants for 8-CH Exchange in Adenine Derivatives.[a]

Temp (°C)	5′-rAMP	cAMP	Poly(rA)
90			0.180
80	0.218	0.221	0.0753
70	0.0787	0.0822	0.0306
60	0.0307	0.0331	0.0115
50	0.00825	0.00942	0.00297
43		0.00504	
40	0.00265	0.00390	0.000776
30	0.000812	0.00152	0.000180
20	0.000190		

[a] Entries are mean values of k_ψ (hr^{-1} units) from two or more determinations on pD 7 solutions of 5′-rAMP (0.25 M), cAMP (0.25 M), and poly(rA) (40 μg/μl). Reproducibility is within ±5% of the mean values cited.

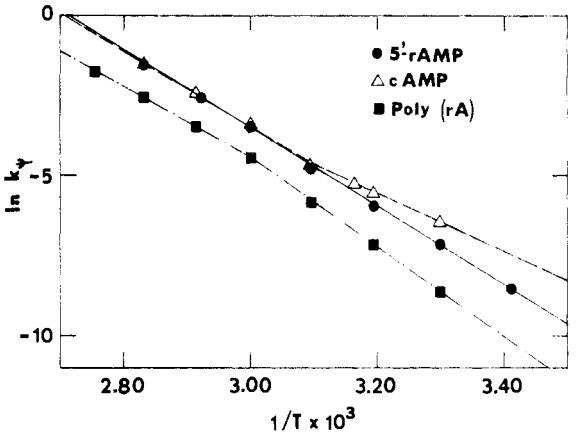


FIGURE 5: Plots of ln k_ψ vs. $1/T$. For cAMP, the straight line in the high-temperature range (low $1/T$) is a least-squares fit to data points at 50°C and higher, and the straight line in the low temperature range (high $1/T$) is a least-squares fit to data points at 50°C and lower. For poly(rA) the two straight lines are least-squares fits to data points at 60°C and higher and at 60°C and lower, respectively. For 5′-rAMP, the line is a least-squares fit to all of the data points.

proaching 5′-rAMP (or cAMP) the poly(rA) curve tapers off and becomes nearly parallel (E_a = 22.0 kcal/mol) to that of 5′-rAMP. The Raman spectra of poly(rA) also show clearly that the present results cannot be due to hydrolysis of the polynucleotide at higher temperatures, to protonation of the bases at lower temperatures, or to other chemical modifications of its molecular structure. Thus even in the absence of base-stacking interactions, 8-CH exchange in poly(rA) is retarded as compared with 5′-rAMP or cAMP and such retardation is evidently the result of steric factors.

Arrhenius parameters derived from the least-squares curves of Figure 5 are listed in Table III. Also included in Table III are Arrhenius parameters for cAMP and poly(rA) that would be obtained if all data points for each compound were fitted to single least-squares straight lines (monophasic curves). However, if such curves are in fact drawn through the data points of Figure 5, deviations are well beyond the estimated limits of error (±5%). Thus, although we have found some agreement with the rate constants derived from tritiation studies (Maslova et al., 1969a; Tomasz et al., 1972; Elvidge et al., 1971, 1973c; Gamble, 1975), the k_ψ values reported here are characterized by considerably lower limits of error and allow important differences to be detected in the temperature dependence of exchange rates in cAMP and poly(rA).

An interpretation of the biphasic curves of Figure 5 is given in the following section.

Conclusions

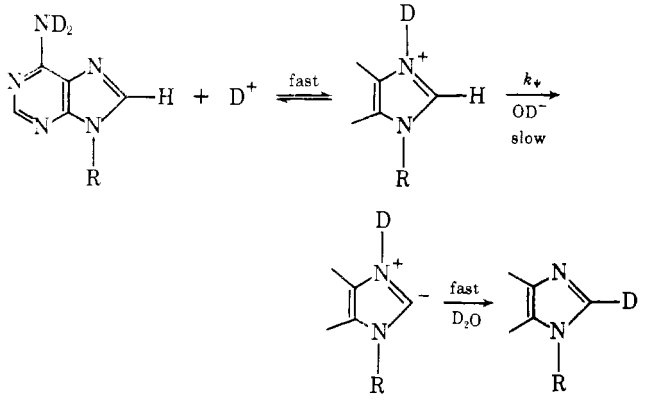
Exchange Kinetics and Molecular Conformation. The exchange kinetics observed for 5′-rAMP, cAMP, and poly(rA) are not sufficiently different from one another or from published data on other adenine derivatives to suggest a mechanism differing from what has already been proposed. The evidence is rather convincing that the C-8 proton is exchanged by way of protonation at the N-7 ring position, followed by abstraction of the C-8 proton to generate an ylide-type intermediate, with subsequent reprotonation at the C-8 position (Tomasz et al., 1972, Elvidge et al., 1973a-c, 1974).

Our results do not conflict with this mechanism, according to which the pseudo-first-order rate constant will exhibit dependence upon the pK value governing protonation at

Table III: Arrhenius Parameters for 8-CH Exchange.

Sample	Activation Energy, E_a (kcal/mol)[c]	Frequency Factor, A (hr^{-1})
5′-rAMP (20–80°C)	24.2 ± 0.6[a]	2.3 × 10^{14} [a]
cAMP (<50°C)	17.7 ± 0.1[a]	9.6 × 10^{9} [a]
(>50°C)	23.5 ± 0.6[a]	8.3 × 10^{13} [a]
(30–80°C)	21.5 ± 1.5[b]	4.3 × 10^{12} [b]
Poly(rA) (<60°C)	27.7 ± 0.4[a]	1.8 × 10^{16} [a]
(>60°C)	22.0 ± 0.1[a]	3.2 × 10^{12} [a]
(30–90°C)	25.3 ± 1.4[b]	3.5 × 10^{14} [b]

[a] Derived from least-squares curves of Figure 5. [b] Derived from a single least-squares curve through all data points (see text). [c] Error limits are calculated from the uncertainties in the slope of the least-squares fitted lines according to the method of Shoemaker et al. (1974).



the N-7 position. For example, a redistribution of the electron density in the adenine ring, which lowers the pK for N-7 protonation, would have the effect of retarding the rate of 8-CH exchange. Such a redistribution is possible and likely for the case of stacking of the bases in poly(rA). The rate of exchange can also be affected by steric factors which do not alter directly the electron distribution in the adenine ring. Electronic and steric effects should be reflected in the slope and intercept, respectively, of the ln k_ψ vs. $1/T$ plots, assuming

$$\ln k_\psi = \ln A - (E_a/RT) \qquad (3)$$

where E_a is the Arrhenius activation energy and A is a temperature-independent frequency factor. Since the deuterium exchange reactions were initiated on samples of uniform isotopic purity, the intercepts of the ln k_ψ vs. $1/T$ plots (Figure 5) provide a realistic basis for comparison of steric factors affecting the exchange in 5′-rAMP, cAMP, and poly(rA).

According to the present results, exchange in 5′-rAMP follows eq 3 strictly over the temperature range 20–80°C. The activation energy (24.2 kcal/mol) and frequency factor (2.3 × 10^{14} hr^{-1}) are consistent with values obtained on other similar CH pseudoacids (Pearson and Dillon, 1953; McKay, 1943; Elvidge et al., 1973c). We conclude therefore that in 5′-rAMP there are neither intermolecular associations (base stacking or otherwise) nor intramolecular conformational changes over the range 20–80°C which alter the lability of the 8-CH bond or its accessibility to solvent molecules. The same conclusion applies to cAMP for the range 50–90°C.

We find, however, that below 50°C, exchange in cAMP is abnormally fast, characterized by a lower activation energy (17.7 kcal/mol) than that governing exchange in 5′-

rAMP. We interpret this result to mean that the electron distribution in the adenine ring of cAMP is altered at lower temperatures so as to facilitate 8-CH exchange as compared with 5′-rAMP. Perturbation of the electronic structure may be due to either inter- or intramolecular factors. However, we observe that the Raman spectrum of cAMP exhibits no significant changes as a function of molecular concentration suggesting that the accelerated exchange rate is more probably due to an intramolecular conformational change than to associative interaction. We therefore conclude further that below 50°C, cAMP is "locked" into a conformation which is characterized by a more labile 8-CH group than is found in 5′-rAMP.

X-Ray diffraction studies indicate that both the syn and anti conformations occur in crystalline cAMP (Watenpaugh et al., 1968). The syn conformation is also found in the cAMP analog adenosine 3′:5′-monophosphonate (Sundaralingam and Abola, 1972). On the other hand, 5′-rAMP, like other 5′-nucleotides, occurs only in the anti form. Therefore the accelerated exchange rate observed for cAMP may very well be due to the occurrence of molecules in the syn conformation, although it is not immediately apparent why syn and anti conformations should differ so significantly in the electronic structure of the adenine ring.

Finally, we interpret the results on poly(rA) as follows. The retardation of exchange below 60°C is attributed primarily to base stacking. This conclusion is not new (Maslova et al., 1969a) but can be reached solely from the present data, since retardation diminishes with unstacking of the bases up to 60°C. This interpretation is also consistent with the fact that base stacking depresses the pK for protonation of the N-7 ring site. Above 60°C, further unstacking of the bases does not further eliminate the retardation of exchange. However, the activation energy governing exchange in poly(rA) above 60°C (E_a = 22.0 kcal/mol) is nearly identical with the value observed for 5′-rAMP and indicates that retardation at high temperature is due to a steric rather than to an electronic effect. We conclude that above 60°C, exchange in poly(rA) is hindered primarily by the polymer backbone which apparently restricts the accessibility of the 8-CH group to solvent molecules.

On the Raman Spectra of Adenine Nucleotides. The results obtained here indicate the usefulness of Raman spectroscopy for studying the kinetics of 8-CH exchange in purines. Measurements of k_ψ as a function of temperature are sufficiently precise to reveal rather small deviations from the behavior predicted by eq 3. The Raman data also provide reliable values of the intercepts in the ln k_ψ vs. $1/T$ plots, whereas these are at best estimated roughly in the tritiation studies.

The spectra of cAMP presented here have also permitted a number of new assignments for the weakly scattering ribosyl groups of the nucleotides. The different patterns of phosphate ester frequencies observed for cAMP and poly(rA) confirm the sensitivity of the P-O stretching vibrations to the conformation of the diester linkages.

Finally, the present results suggest the following comments on recent normal coordinate calculations for 9-methyladenine (Tsuboi et al., 1973). The 8-CH stretching vibration is observed here at 3120 cm^{-1}, supporting the calculated frequency of 3131 cm^{-1} in 9-methyladenine. The observation that C-8 deuteration shifts many of the adenine ring frequencies (Figure 3) supports the refined treatment of Tsuboi et al. (1973), in which the ring skeletal motions cannot be dissociated from the 2-CH and 8-CH deformations. We find, however, that the in-plane 8-CH deformation contributes substantially to Raman lines of 5′-rAMP and cAMP near 1320, 1380, and 1340 cm^{-1}, which does not appear to be the case for 9-methyladenine. We conclude that the force field applied to 9-methyladenine cannot be transferred directly to adenine nucleotides. This is most likely due to the fact that methyl carbon–ring nitrogen (1′C–9N) stretching in 9-methyladenine generates a different pattern of normal modes than that resulting from ribosyl carbon–ring nitrogen stretching in adenine nucleotides. In this connection it seems worthwhile to mention that the Raman spectra of 5′-rAMP and cAMP, though very similar as regards adenine ring frequencies, are not identical. This fact is most clearly revealed by comparing the half-width and peak height of the Raman line at 720 cm^{-1} in 5′-rAMP with the counterpart in cAMP. The observed differences can be accounted for if it is assumed that this adenine ring vibration involves some small but significant contribution from the stretching and bending motions of the 1′C–9N group which is different for 5′-rAMP and cAMP by virtue of their different ribosyl ring structures.

In summary, Raman spectroscopy offers several advantages over tritium labeling techniques for the precise determination of rate constants governing isotopic hydrogen exchange in purines. By monitoring the appropriate Raman lines it is also possible, in principle, to determine simultaneously the different rates of exchange of nonidentical purine residues in a biopolymer, such as the adenine and guanine residues of DNA or tRNA.

Acknowledgments

We are grateful to Dr. R. C. Gamble and Professor P. R. Schimmel for discussing with us their tritiation studies prior to publication.

References

Bullock, F. J., and Jardetzky, O. (1964), *J. Org. Chem. 29,* 1988–1990.

Chen, M. C., and Thomas, Jr., G. J. (1974), *Biopolymers 13,* 615–626.

Doppler-Bernardi, F., and Felsenfeld, G., (1969), *Biopolymers 8,* 733–741.

Eidinoff, M. L., and Knoll, J. E. (1953), *J. Am. Chem. Soc. 75,* 1992–1993.

Elvidge, J. A., Jones, J. R., O'Brien, C., and Evans, E. A. (1971), *Chem. Commun.,* 394–395.

Elvidge, J. A., Jones, J. R., O'Brien, C., Evans, E. A., and Sheppard, H. C. (1973b), *J. Chem. Soc., Perkin Trans. 2,* 1889–1893.

Elvidge, J. A., Jones, J. R., O'Brien, C., Evans, E. A., and Sheppard, H. C. (1973c), *J. Chem. Soc., Perkins Trans. 2,* 2138–2141.

Elvidge, J. A., Jones, J. R., O'Brien, C., Evans, E. A., and Sheppard, H. C. (1974), *J. Chem. Soc., Perkin Trans. 2,* 174–176.

Elvidge, J. A., Jones, J. R., O'Brien, C., Evans, E. A., and Turner, J. C. (1973a), *J. Chem. Soc., Perkin Trans. 2,* 432–435.

Englander, S. W. (1963), *Biochemistry 2,* 798–807.

Fritzsche, H. (1967), *Biochim. Biophys. Acta 149,* 173–179.

Gamble, R. C. (1975), Ph.D. Thesis submitted to M.I.T., Department of Chemistry, Cambridge, Mass.

Gamble, R. C., and Schimmel, P. R. (1974), *Proc. Natl.*

Acad. Sci. U.S.A. 71, 1356–1360.
Hartman, K. A., Lord, R. C., and Thomas, Jr., G. J. (1973), in Physico-Chemical Properties of Nucleic Acids, Vol. 2, Duchesne, J., Ed., New York, N.Y., Academic Press.
Lafleur, L., Rice, J., and Thomas, Jr., G. J. (1972), *Biopolymers 11,* 2423–2437.
Livramento, J., and Thomas, Jr., G. J. (1974), *J. Am. Chem. Soc. 96,* 6529–6531.
Lord, R. C., and Thomas, Jr., G. J. (1967), *Spectrochim. Acta, Part A 23,* 2551–2591.
Maslova, R. N., Lesnick, E. A., and Varshavsky, Ya. M. (1969a), *Biochem. Biophys. Res. Commun. 34,* 260–265.
Maslova, R. N., Lesnick, E. A., and Varshavskii, Ya. M. (1969b), *Mol. Biol. (Engl. Transl.) 3,* 728–737.
McConnell, B., and Seawell, P. C. (1972), *Biochemistry 11,* 4382–4392.
McDonald, C. C., Phillips, W. D., and Penswick, J. (1965), *Biopolymers 3,* 609–616.
McKay, H. A. C. (1943), *J. Am. Chem. Soc. 65,* 702–706.
Medeiros, G. C., and Thomas, Jr., G. J. (1971), *Biochim. Biophys. Acta 247,* 449–462.
Osterman, L. A., Adler, V. V., Biblaschvili, R., Savochkina, L. P., and Varshavskii, Ya. M. (1966), *Biokhimiya 31,* 398–404.
Pearson, R. G., and Dillon, R. L. (1953), *J. Am. Chem. Soc. 75,* 2439–2443.
Prescott, B., Gamache, R., Livramento, J., and Thomas, Jr., G. J. (1974), *Biopolymers 13,* 1821–1845.
Rimai, L., Cole, T., Parsons, J. L., Hickmott, Jr., J. T., and Carew, E. B. (1969), *Biophys. J. 9,* 320–329.
Schweizer, M. P., Chan, S. I., Helmkamp, G. K., and Ts'o, Sundaralingam, M. (1969), *Biopolymers 7,* 821–860.
Sundaralingam, M. (1973), Proceedings of the Vth Jerusalem Symposium on Quantum Chemistry and Biochemistry, pp 417–456.
Sundaralingam, M., and Abola, J. (1972), *Nature (London) 235,* 244–245.
Thomas, Jr., G. J. (1970), *Biochim. Biophys. Acta 213,* 417–423.
Thomas, Jr., G. J., and Barylski, J. R. (1970), *Appl. Spectrosc. 24,* 463–464.
Thomas, Jr., G. J., and Hartman, K. A. (1973), *Biochim. Biophys. Acta 312,* 311–322.
Tomasz, M., Olson, J., and Mercado, C. M. (1972), *Biochemistry 11,* 1235–1241.
Tsuboi, M., Takahashi, S., and Harada, I. (1973), in Phyiico-Chemical Properties of Nucleic Acids, Vol. 2, Duchesne, J., Ed., New York, N.Y., Academic Press.
Yu, Nai-Teng (1969), Ph.D. Thesis, Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Mass.
Watenpaugh, K., Dow, J., Jensen, L. H., and Furberg, S. (1968), *Science 159,* 206–207.
P. O. P. (1964), *J. Am. Chem. Soc. 86,* 696–700.
Searcy, D. G. (1968), *Biochim. Biophys. Acta 166,* 360–370.
Shelton, K. R., and Clark, J. M., Jr. (1967), *Biochemistry 6,* 2735–2739.
Shimanouchi, T., Tsuboi, M., and Kyogoku, Y. (1964), *Adv. Chem. Phys. 6,* 435–498.
Shoemaker, D. P., Garland, C. W., and Steinfeld, J. I. (1974), Experiments in Physical Chemistry, 3rd ed, New York, N.Y., McGraw-Hill, p 47.

Chemical Synthesis of N^{β}-Oxalyl-L-α,β-diaminopropionic Acid and Optical Specificity in Its Neurotoxic Action†

S. L. N. Rao

ABSTRACT: A practical procedure is described for the bulk synthesis of the neurotoxin N^{β}-oxalyl-L-α,β-diaminopropionic acid (OA_2pr^3), a potential dicarboxylic amino acid antagonist of *Lathyrus sativus* seeds. L-Aspartic acid was reacted with sodium azide in 30% fuming sulfuric acid and L-α,β-diaminopropionic acid hydrochloride (A_2pr^3·HCl) was isolated in yields greater than 75%. Potassium methyl oxalate was found to react selectively with the β-amino group of A_2pr^3 resulting in near quantitative yields of OA_2pr^3. D-OA_2pr^3 has been made for the first time by this procedure. Unlike L-OA_2pr^3 the naturally occurring neurotoxin, D-OA_2pr^3, is not neuroactive even in high doses. The microsynthesis of L-[2,3-^{3}H]A_2pr^3 from L-[2,3-^{3}H]aspartic acid is also described, and the same procedure could also be used to prepare the neurotoxin with other labels. The availability of the neurotoxin in bulk and in labeled form should further experimental approaches to the understanding of its mechanism of action.

N^{β}-Oxalyl-L-α,β-diaminopropionic acid (OA_2pr^3)[1] is the major neurotoxic amino acid isolated from the seeds of *Lathyrus sativus* (Rao et al., 1964; Murti et al., 1964) and detected in several other species of *Lathyrus* (Bell and Donovan, 1966). Recent studies indicate that OA_2pr^3 is a potential antagonist of the dicarboxylic amino acids (Mehta et al., 1972; Magalhaes and Packer, 1972; Laxmanan and Padmanaban, 1974). Despite the well documented neurotoxic property of OA_2pr^3 (Nagarajan et al., 1965; Rao et al., 1967; Rao and Sarma, 1967) and its effects on certain biological systems (Cheema et al., 1970, 1971; Mehta et al.,

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[1] Abbreviations used are: A_2pr^3, α,β-diaminopropionic acid; OA_2pr^3, N^{β}-oxalyl-L-α,β-diaminopropionic acid.