THE TAUTOMERIC EXCHANGE OF NUCLEIC ACID BASES

Thesis by

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SUMMARY

The thesis for my Master of Science degree contains two topics, i. e., mainly on the study of the tautomeric exchange of nucleic acid bases, Cytosine and Guanosine.

By proton magnetic resonance study, I have established that these two bases do exist in their minor tautomeric forms with significant percentage. This is obviously contrary to Watson and Crick's original hypothesis of genetic mutation. A new theory should therefore be proposed to meet this discovery. The abstract of the results of this study will be given before each topic.

The Tautomeric Exchange of Nucleic Acid (I)

Cytosine

Abstract

For a series of PMR studies of cytidine and related compounds in D_2O solution at various pD, it was observed that the H_5 resonances of cytosine base are broad in some of these compounds at pD 4-7, while those of H_6 are not. We proposed that there is chemical exchange between two tautomeric forms of cytosine base to give rise to broadening of spectra. This chemical exchange was found to be catalyzed by both solvent (D_2O) and hydroxyl (OD^-) ion present in solution. The observation of sharp resonances in certain cytidine derivatives having only one possible tautomeric structure supports this proposal. Temperature study of H_5 resonances at different pD was used to investigate the kinetics of this exchange reaction. The percentage of its minor tautomer was estimated as $10 \pm 2\%$ in neutral aqueous solution and its life time is about 10^{-3} sec at room temperature. The activation energy of this tautomeric exchange is 7.5 ± 0.5 kcal/mole. for D_2O catalysis and 2.5 ± 0.5 kcal/mole for OD^- catalysis. The relation between tautomeric exchange of nucleic acids and genetic mutation is also discussed.

Introduction

The transfer of genetic information during the replication of DNA relies on the unique pairing of the complementary nucleic acid bases.[1,2] It has, however, been shown that these bases exist in other tautomeric forms in addition to the principal tautomeric structure, and the presence of these minor base tautomers has been postulated as an important source of the imperfect pairing which can lead to genetic mutation.[3] The cytosine base, for example, may exist in either its normal amino or an abnormal imino form.[4,5] It is well known that the normal amino form pairs with guanine to form a stable Watson-Crick G-C base-pair; the imino tautomer, by contrast, has the appropriate electronic structure for pairing with the adenine base to form the ab-
1) normal A-C base-pair. In view of the important biological implications of the above tautomerism, it is desirable to obtain a detailed understanding of the thermodynamic and kinetic properties of the amino-imino system. The possibility of this tautomerism has been examined by uv spectrophotometric studies of cytosine and its derivatives, and more recently, there have been some theoretical attempts by Pullman and Löwdin to predict the existence of this tautomerism and to ascertain the energetics involved.[6-8] In spite of these efforts, our understanding of the problem is still far from satisfactory, and in particular, experimental data are lacking on the kinetics of the tautomerism.

It is shown in this paper that proton magnetic resonance spectroscopy can be an effective method for the examination and the elucidation of the amino-imino tautomerism in cytosine. It has frequently been observed that the H_5 resonance in the pmr spectra of

cytosine and related compounds in aqueous solution exhibits unusual broadening under certain conditions of temperature and pH.[9] This line broadening can be shown to arise from tautomeric exchange between the amino and imino species, and from a detailed study of the temperature and pH dependences of the H_5 line-broadening, we have been able to determine the equilibrium and dynamical properties of this tautomerism.

Experimental

A number of cytosine nucleosides and nucleotides were selected for this study. These compounds were obtained from various sources: Cytosine and cytidine were purchased from Calbiochem, Los Angeles, Calif.; 5'-cytidine monophosphate (5'-CMP) was obtained from Sigma Chemical Co., St. Louis, Mo.; 2'-CMP and 3'-CMP were obtained from P-L Biochemicals, Inc., Milwaukee, Wis.; and 3-methyl cytidine (methosulfate salt) was obtained from Mann Research Laboratory, New York, N.Y. The N, N'-dimethylcytosine derivative was synthesized from 4-ethoxy-2-hydroxyl pyrimidine provided by Cyclochemical Corp., according to the procedure of Wempen et al.[10]

A column of Dowex 50W-X8 cation exchange resin was used to convert the various nucleotides to their sodium salts and to remove paramagnetic ion impurities. In the case of 5'-CMP, the absence of impurities at significant levels after ion exchange was confirmed by atomic absorption analysis of the sample by Schwarzkope Microanalytical Laboratory, Woodside, N.Y.

Sample solutions were prepared at concentrations of 0.01 to 0.05 M in 99.7% D_2O supplied by Columbia Organic Chemicals (Columbia, S.C.), and the compounds were sometimes lypholized from D_2O prior to sample preparation in order to reduce the magnitude of the HDO peak in the spectra. The pD of each solution was measured with a Leeds and Northrup 7401 pH meter, equipped with miniature electrodes, and was taken to be the observed pH meter reading plus 0.4 (the standard correction). pH adjustments were made with either 1M DCl or 1M NaOD.

The pmr spectra of the various nucleoside and nucleotide solutions were recorded on Varian HA-100 and HR-220 spectrometers. A C-1024 time-average computer was used to enhance the signal-to-noise ratio. To simplify the measurement of the cytosine H_5 resonance widths, the normal H_5 doublet was frequently collapsed by spin decoupling to remove complications arising from the spin-spin coupling between the H_5 and H_6 protons. Chemical shifts were measured relative to an external TMS capillary, which also provided the field/frequency lock signal for HA-100 operation. The error in the measurement of the chemical shifts and linewidths is 0.5 Hz. The ambient probe temperature was 30±1°C in the case of the HA-100 spectrometer and 17±1°C for the HR-220. In the variable-temperature studies, the sample temperature was controlled to±1°C by means of a variable temperature controller and was determined using the methanol and ethylene glycol samples and calibration curves supplied by Varian.

Results

The pmr spectra of cytosine and many of its nucleoside and nucleotide derivatives have previously been reported and discussed.[9,11] In our present work, we shall primarily be concerned with the spectral behavior of those resonances due to the H_5 and H_6 protons of the cytosine base. These resonances appear as doublets due to the spin-spin coupling between these protons and are readily identified by the spin-spin splitting of 8 Hz. The H_5 doublet is found 6 ppm downfield from TMS, and the H_6 doublet 8 ppm downfield from TMS. The H_6 resonances are always well-defined and relatively sharp; by contrast, the H_5 resonances are frequently appreciably broadened, often so much as to escape detection. This H_5 linebroadening is both pH and temperature dependent. The elucidation of this heretofore unexplained linebroadening constitutes the principal objective of this work, and we now summarize the results of a systematic study which we have undertaken to characterize this phenomenon.

pH Studies. As illustrated in Figures (2) and (3), the spectral positions of the cytosine H_5 and H_6 proton resonances of cytosine and its nucleoside and nucleotide derivatives are pD dependent. The chemical shifts of these protons, for example, change abruptly with pH in the pD region 4-5, where ring protonation is known to occur. Above pD 5, however, the chemical shifts are relatively independent of pD, except in the case of 5'-CMP, where effects due to ionization of the phosphate group at pD 6 are apparent. In pyrimidine nucleotides, the conformation of the base about the glycosidic bond is expected to be anti, and in this

base conformation, the phosphate group is in juxtaposition with that part of the cytosine base bearing the H_5 and H_6 protons. As expected, the deshielding effect of the negatively charged phosphate group is more pronounced for the H_6 than the H_5 proton because of the closer proximity of the phosphate group to the H_6 proton.

In contrast to the essentially constant chemical shifts, the linewidth of H_5 resonance is strongly pD dependent in the pD range 4-7, exhibiting a maximum near pD 6. The width of H_6 resonance is also pD dependent, but to a much less extent.

The $H_{1'}$ ribose resonance also remains sharp throughout the pH range investigated. As shown in Figure (4), the extent of broadening of the H_5 resonance as well as the pH range over which the broadening is manifested depends somewhat on the compound examined. We shall, therefore, discuss each case separately.

(1) Cytosine:

In this case, the linewidth of H_5 resonances increases abruptly in the pD range 3-5, reaching a maximum of 11 Hz at pD 5.5 and then decreasing to a width of 2 Hz at pDs 8 and above. Over the same pD range, the H_6 resonances behave qualitatively the same but reach a maximum of only 4 Hz at pD 5.

(2) Cytidine:

In spite of the addition of the ribose moiety, the pD dependence of the linewidth of both H_5 and H_6 resonances is similar to that of cytosine as can be seen from Figure (4). The maximum H_5 linewidth is about 10 Hz at pD 5.8, and the H_6 broadening is again significantly less. The ribosal $H_{1'}$ resonance which was also observed does not appear to broaden.

(3) 5′-CMP, 3′-CMP, 2′-CMP:

The addition of the phosphate group to the nucleoside as described above again has little effect on the qualitative behavior of H_5 linewidth. However, the absolute maximum obtained and its corresponding pD are changed slightly with both the presence of a phosphate group and the position of that phosphate group on the ribose ring. In the case of 5′-CMP the maximum linewidth of H_5 resonance obtained is 8 Hz at pD 6, the H_6 linewidth for this nucleotide reaches a maximum only about 3 Hz at the same pD. In 3′-CMP the maximum H_5 linewidth is about 13 Hz at pD 6, while in 2′-CMP the maximum width of H_5 is only 5 Hz. For the latter two nucleotides the H_6 resonance is again broadening to a much lesser extent than the H_5 resonance.

(4) 4-NN′ dimethyl cytosine, 3-methyl cytidine methosulfate.

In these compounds, which have only one possible neutral tautomeric structure, amino form in the case of 4-NN′ dimethyl cytosine and imino form in the case of 3-methyl cytidine, both the H_5 and H_6 resonances of the pyrimidine base remain sharp throughout the pD range of interest. Although the linewidth was studied from pD 2 to 12, for comparative purposes, only the range in which the neutral tautomeric form exists is significant. In the case of 4-NN′ dimethyl cytosine, this range is essentially that of normal cytosine base (pD 4-10), however, in the case of 3-methyl cytidine the neutral species predominates only in a narrow pD range beginning with pD 7.5, at which the amino proton is lost to form an imino tautomer and ending near pD 9.5, where the second base proton is lost. The existence and values of the two pDs involved in this process are apparent from the width of the pD titration

curve. The values of 8.2 and 10.5 were confirmed by an analysis of UV and NMR pD curves.

(5) CpC, 2′-deoxy cytidine, and 2′-deoxy CMP:

The same study was carried out in these compounds and similar line-broadening of H_5 resonance of their cytosine base was also observed. The missing of 2′ OH group on the sugar moiety in the 2′-deoxy nucleoside and 2′-deoxy nucleotides was found to have no significant effect on this phenomenon.

Temperature Study. The investigation of the temperature dependence of the linewidth of H_5 resonance of some of these compounds was carried out in the solutions with slightly different pD between 90°C and -25°C. For the study of 5′-CMP, the solutions containing saturated sodium chloride were prepared, while the pure solutions were used in the temperature study of cytidine carried out between 30°C and 3°C. The result of this study was shown in Figures (6) and (7). The general feature of this study is that there is a linewidth maximum of H_5 resonance at certain temperature depending slightly on the solution pD. Beyond this, the H_5 linewidth becomes sharper either up to the higher temperature or down to the lower temperature. As shown in Figure (7), the H_5 linewidth of 5′-CMP at pD 6.5 is about 7.8 Hz at 30°C, but it becomes 5.2 Hz at 45 °C and 2.3 Hz at 80°C, while at temperature lower than 30°C it becomes broader and reaches its maximum 11.5 Hz at 5°C. At temperatures below this, the H_5 linewidth sharpens again with decrease in temperature, until it is reduced to 5 Hz at -20°C. Similar result was obtained for the study of cytidine, and it is shown that the pD of the solution giving the same H_5 linewidth is a little shifted by 0.4 units

due to the presence of high concentration of salt.

Field Dependence Study. Under the same conditions, by comparing the linewidth of H_5 resonance of 5'-CMP at pD 5.2 measured with different external field, strong field dependence of the H_5 linewidth was observed. Thus the linewidth measured by HR-220 is much broader than that measured by HA-100 at 16°C, ie;

$\Delta\nu$ = 16.5 Hz measured by HR-220.

$\Delta\nu$ = 6.5 Hz measured by HA-100.

Discussion

Broadening of the H_5 resonances of cytosine and its derivatives in aqueous solution could arise from a number of sources. These include contamination by paramagnetic ions, spin coupling of H_5 proton with neighboring quadrupolar N^{14} nitrogens, an increase in the degree of cytosine aggregation, or some chemical exchange processes occurring with the NMR time scale.[12-14]

The presence of paramagnetic ions in the sample solution could drastically reduce the spin-lattice relaxation time for the H_5 proton due to the strong coupling between the unpaired electron spin and the spin of the resonant nuclei. In fact, it was experimentally shown that in the presence of 10^{-4} N Mn^{++} ions, the H_5 resonance of 2'-CMP was broadened in the pD range 4 to 7. However in contrast to the selective broadening of the H_5 resonance observed in our study, it was noted that the broadening due to the presence of Mn^{++} was rather indiscriminate leading to broadening of the H_6 and $H_{1'}$ resonances as well. This fact together with our

extensive efforts to eliminate the possible paramagnetic ion contaminants from our solution led us to conclude that this broadening mechanism is not of major concern in our case.

The quadrupole moment of N^{14} amino nitrogen could also provide an additional relaxation path for its spin-spin coupled H_5 proton. However the absence of any H_5 broadening in either the NN-dimethyl cytosine or 3-methyl cytidine derivative, which exemplifies the two major tautomeric structures for a cytosine base, strongly discounts this possibility. This mechanism seems also unlikely in the light of relatively small H_5-N^{14} spin-spin coupling.

Nucleotides and nucleosides, especially those of the purine base have been shown to aggregate in the form of vertical stack in aqueous solution. If such association occurred in the case of cytosine, one might expect the increased rotational correlation time for the aggregates leading to a reduction in spin-lattice relaxation time and thus abroader H_5 resonance. However, the absence of a change in chemical shift or linewidth of cytosine protons as a function of concentration in the range 0.01 to 0.1 M, indicates a relatively small degree of association in this concentration range, and a contribution to H_5 linewidth through this mechanism seems not reasonable.

The elimination of the above possibilities leaves only a chemical exchange phenomenon as the source of H_5 line broadening in cytosine. Generally speaking when the magnetic environment of the resonance proton is changed at a rate comparable to the NMR time scale, the transverse relaxation time is shortened to the point of the linewidth increase. When the temperature of the solution rises, the rate of the exchange is expected to increase, likewise a decrease in temperature causes a corresponding

decrease in the rate of exchange. As the exchange rate departs from that of the nmr experiment one expects that the observed linewidth returns to the normal natural linewidth limit. This is in fact in agreement with our observation as presented in Figure (6), and confirms our interpretation of line broadening as a chemical exchange phenomenon. From the temperature study of 5′-CMP at 16°C, it is apparent that the exchange rate is on the fast exchange side of our experimental time scale. In this case the linewidth dependence on chemical exchange rate between species A and B can be expressed as follows:

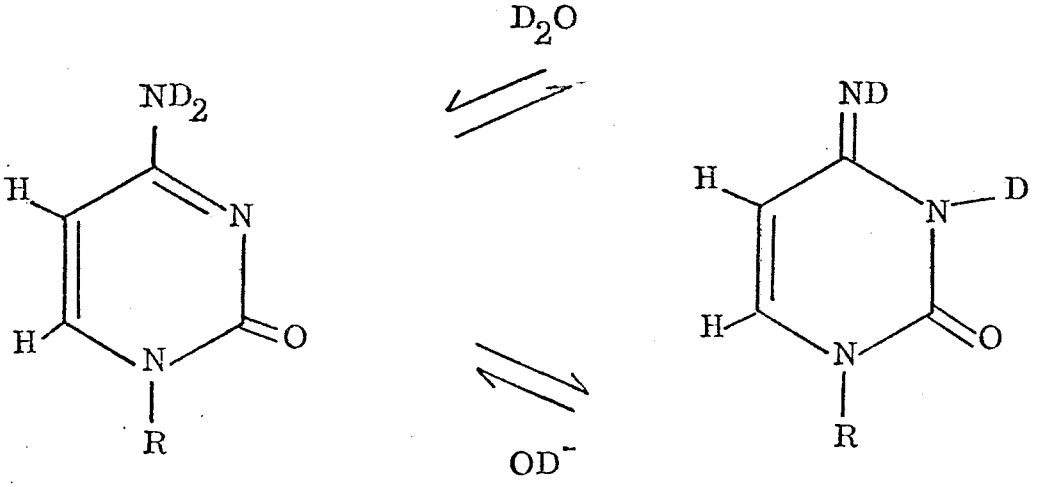
$$\nu_{obs} = \nu_0 + \frac{1}{\pi} P_A^2 P_B^2 (\dot{\omega}_A - \dot{\omega}_B)^2 (\tau_a + \tau_b) \qquad (1)$$

where ν_{obs} and ν_0 are the observed linewidth and natural linewidth respectively, P_A and P_B are their respective populations, $\dot{\omega}_A$ and $\dot{\omega}_B$ are the chemical shifts of these two species respectively, and τ_a, τ_b denote their life time during the exchange.

Since $\dot{\omega}_A$ and $\dot{\omega}_B$ are directly proportional to the field strength Eq. (1) predicts a direct square dependence of the linewidth on the magnetic field. This is in fact in agreement with out observation of 5′-CMP at 16°C and pD 5.2 where its H_5 linewidth increased by a factor of 3 in going from 24000 (at HA-100) to 53000 (HR-220) gausses of external magnetic field. Thus we can quite definitely conclude that we are observing the chemical exchange effect.

The actual change in magnetic environment can occur in a number of ways, but the evidence presented here is strongly towards the exchange between tautomers of the neutral cytosine base. The fact

that line broadening is observed in the nucleoside and simple cytosine base as well as nucleotides indicates that neither the phosphate group nor the ribose moiety are necessary for the exchange process. The absence of a cytosine concentration dependence points to the fact that the exchange process is unimolecular in cytosine and its derivatives. And the absence of the line broadening in 4 NN' -deimethyl cytosine and 3-methyl cytidine where either the neutral imino or amino tautomer is eliminated confirms the involvement of these two structures in the exchange process. We therefore proposed the following tautomeric exchange to be responsible for the observed effect.

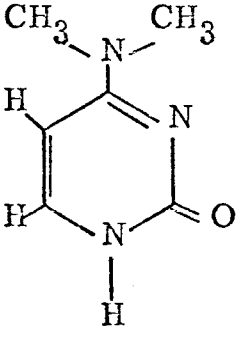


We shall show this model to explain the observed temperature and pD dependence of the H_5 linewidth and can be used to derive certain thermodynamic and kinetic parameters.

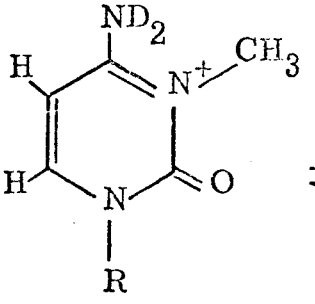
From the chemical shift data of 3-methyl cytidine at various pD, we may easily calculate the chemical shifts of H_5 and H_6 resonances of 3-methyl, 4-imino cytidine which only exists stably near pD 8.5 and may roughly be taken as imino cytidine. By comparing these with the shifts of the corresponding resonances of normal cytidine at the same pD, we may estimate that the chemical shift difference of H_5 resonances between amino and imino cytidine is about 16 Hz, while that of H_6 is only 5 Hz. From Eq. (1), we know that in the limit of fast exchange between amino and imino tautomers, the exchange linewidth ($\Delta\nu_{obs} - \Delta\nu_0$) would be proportional to the square of the chemical shift difference between two tautomers, ie;

$$\Delta\nu_{obs} - \Delta\nu_0 \quad \alpha \quad (\dot{\omega}_A - \dot{\omega}_B)^2$$

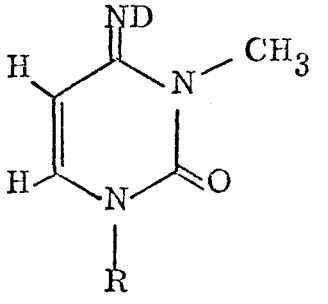
From this relation, we might predict that the linewidth of H_5 resonance of cytidine would be at least 8 times broader than that of H_6. This would explain why we observed only broadening of H_5 resonance but not H_6 and support further this amino-imino tautomeric exchange.



4 NN′ Dimethyl cytosine



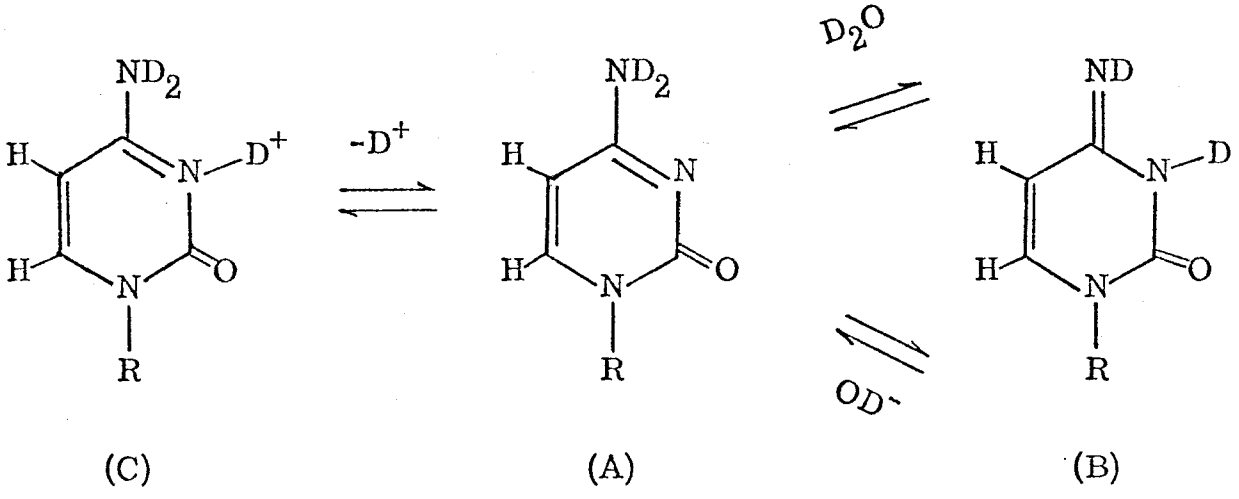
3-methyl cytidine



$7.5 < pD < 9.5$
3-methyl 4-imino cytidine

Kinetic Analysis

Having established that the linewidth behavior observed for these compounds is the result of tautomeric exchange, it is possible to extract from the temperature and pD dependence of linewidths certain thermodynamic and kinetic parameters for the process. However, this type of quantitative treatment requires first the development of an accurate theoretical equation, since our data extend over a broad range of pD and temperature. We must include the effect of exchange with protonated form as well as two neutral tautomers, ie; the process described in the following scheme:



(C) (A) (B)

where C, A, and B represent the protonated, amino and imino tautomers respectively.

In addition to the necessity of considering three sites exchange we must develop an equation applicable over a broad range, ie; for the fast, intermediate and slow exchange. Thus in appendix I we have begun from the modified Bloch equations in an attempt to derive the necessary

formulae. The following expression for the observed linewidth was derived to describe the three sites exchange

$$\Delta\nu\,\text{obs} = \left(\frac{1}{\pi\,T_2}\right)_{\text{obs}} = \Delta\nu_0 + \frac{P_B^{\,2}}{\pi\,P_A}\,(1-P_B)^2\,(\omega_{AC}-\omega_B)^2\,\tau_{ab} \qquad (2)$$

where $\Delta\nu\,\text{obs}$ is the observed linewidth. $\Delta\nu_0$ is the natural linewidth in the absence of exchange. P_A, P_B are populations of animo and imino tautomers of cytosine base. ω_{AC} is the average resonance frequency of protonated and neutral cytosine base. ω_B is the resonance frequency of imino cytosine. τ_{ab} is the mean life time of amino tautomer exchanged into its imino tautomer.

Consider the various expression for the linewidth derived from Eq. (1)[2] under different pDs and temperatures.

At pD $>$ 4, there is significant amount of protonated species (C) which is assumed to exchange very fast with the neutral cytosine only, and have no contribution to H_5 line broadening. However the chemical exchange between tautomers is supposed to be a relatively slow process and does give some broadening effect on the H_5 linewidth. Since the exchange rate is still on the fast exchange side at room temperature, ie; Eq.(2) is adequate to describe the linewidth of H_5 resonance.

Based on the experimental observation of H_5 linewidth at different pDs and temperatures, we may assume that the chemical exchange between two tautomers is involved in withdrawing of proton from amino group either by solvent (D_2O) or OD^- and then rearranging to form the imino tautomer. Hence it is assumed that there are two paths through-

which the tautomeric exchange proceeds, ie; one is catalyzed by solvent, (D_2O) and the other is by OD^-.

Based on this assumption, we may find a direct relation between the observed linewidth and the exchange rate by the following analysis:

$$-\left(\frac{d[A]}{dt}\right)_f = k_h^a [A][OD^-] + k_s^{a'} [A][D_2O]$$

$$= k_h^a [A][OD^-] + k_s^a [A] \qquad (3)$$

Eq.(3) gives the exchange rate of amino tautomer (A) into imino form (B). where $[A]$ $[OD^-]$, $[D_2O]$ are the respective concentrations of amino cytosine, deuteroxyl ions and solvent. $[D_2O] \approx 55.1$ mole $\approx$ const. in dilute solution.

From Eq. (3) we can get the expression for the mean life time of species A,

$$\frac{1}{\tau_{ab}} = -\frac{\left(\frac{d[A]}{dt}\right)_f}{[A]} = k_h^a [OD^-] + k_s^a$$

$$= k_1^a e^{-E_h^a/RT} [OD^-] + k_2^a e^{-E_s^a/RT} \qquad (4)$$

Where E_s^a and E_h^a represent the activation energy by each path respectively. Where $k_h^a = k_1^a e^{-E_h^a/RT}$ $k_s^a = k_2^a e^{-E_s^a/RT}$.

Similar rate and life time expression for the minor imino form B can also be derived by the same treatment.

Since the resonance of the imino tautomer cannot be observed at low temperatures when the resonances of two tautomers are separated, we may conclude that the percentage of the imino tautomer is much less

than that of the amino tautomer. (Fig. 10)

From the deprotonation constant, and energy difference of the two tautomers ΔH, assuming that their entropy difference can be neglected, the populations of the two tautomers as a function of deuterium ion concentration can be obtained;

$$P_A + P_B + P_C = 1$$

$$\frac{P_A}{P_C}[D^+] = Kd$$

$$\frac{P_A}{P_B} = e^{\Delta G/RT} \approx e^{\Delta H/RT} \tag{5}$$

$$P_B = \left\{1 + \left[1 + \frac{[D^+]}{k_d}\right] e^{\Delta H/RT}\right\}^{-1}$$

$$\approx \left[\left(1 + \frac{[D^+]}{k_d}\right) e^{\Delta H/RT}\right]^{-1} \tag{6}$$

By substituting Eq. (4), (5), (6) into Eq. (2), and assuming that $P_a \gg P_b$ we obtain the following expression for the linewidth as a function of both pD and temperature.

$$(\Delta\nu)_{obs} = \Delta\nu_0 + k(\dot{\omega}_{AC} - \dot{\omega}_B)^2 \, e^{(E_s^a - 2\Delta H)/RT} \frac{[D^+]}{\left\{1 + \frac{[D^+]}{k_d}\right\}\left\{[D^+] + k_3 k_w \, e^{(E_s^a - E_h^a)/RT}\right\}} \tag{7}$$

where $k = \frac{1}{\pi k_2^a}$ $k_3 = \frac{k_1^a}{k_2^a}$ $k_w \approx 10^{-14}$, is the ionization constant of D_2O.

By taking $\frac{d \ln (\Delta\nu - \Delta\nu_0)}{d[D^+]} = 0$, we can find the concentration of

deuterium ion at which the observed linewidth is a maximum. Experimentally it was found that the maximum H_5 linewidth occurs at pD-6 for 5′ CMP in room temperature study. By substituting this into the equation derived, we can get that

$$k_3 = 4\times 10^{+7}\ e^{(E_h^a - E_s^a)/0.6}$$

The theoretical calculation using Eq. (7) by computer simulation for the pD dependence of linewidth of H_5 resonance is shown in the solid curve of Fig.(9) which almost agrees with the experimental data.

As shown in the Eq. (7), when the $[D^+]\rightarrow 0$ at higher pD the second term of the Eq. (7), vanishes, the observed linewidth approaches the natural linewidth limit ($\Delta\nu_0$).

At lower temperature, when the tautomeric exchange goes through the slow exchange limit, ie; $\frac{1}{\tau_b} \ll (\dot{\omega}_a - \dot{\omega}_b)$, the H_5 resonance of two tautomeric species are separated. The linewidth of the major amino tautomer is therefore given by the following expression which may easily be derived from:

$$\begin{aligned}\Delta\nu_A &= \Delta\nu_0 + \frac{1}{\pi}\frac{1}{\tau_{ab}} \\ &= \Delta\nu_0 + \frac{1}{\pi}\{k_2^a\ e^{-E_s^a/RT} + k_1^a\ e^{-E_h^a/RT}\,[OD^-]\} \qquad (8) \\ &= \Delta\nu_0 + \frac{k_2^a}{\pi}\{e^{-E_s^a/RT} + k_3\ e^{-E_h^a/RT}\,[OD^-]\}\end{aligned}$$

As shown in this equation the observed linewidth at slow exchange side is linearly dependent on the OD^- in the solution at the same temperature. Since the linewidth is very sensitive to the changes in solution pD, it is better to calculate these thermodynamic and kinetic parameters from the observed linewidth at different temperatures in the same pD, ie;

$$\frac{(\Delta\nu_A - \Delta\nu_o)T=T_1}{(\Delta\nu_A - \Delta\nu_o)T=T_2} = \frac{\frac{1}{(\tau_{ab})\,T=T_1}}{\frac{1}{(\tau_{ab})\,T=T_2}} = \frac{\{e^{-E_s^a/RT_1} + k_3\, e^{-E_h^a/RT_1}\,[OD^-]_o\}}{\{e^{-E_s^a/RT_2} + k_3\, e^{-E_h^a/RT_2}\,[OD^-]\}} \qquad (9)$$

where $k_3 = \frac{k_1^a}{k_2^a} = 4 \times 10^7\ e^{(E_h^a - E_s^a)/0.6}$

Hence we have a set of exponential equations. By simply measuring the linewidth in the slow exchange limit at various temperatures and solving these equations by computer graphical method, we can easily obtain the rate constants and activation energies; E_h^a, E_s^a.

According to the above kinetic analysis, it was calculated that the activation energy by solvent catalysis is 7.5 ± 0.5 Kcal/mole, while it is only 2.5 ± 0.5 Kcal/mole by OD^- catalysis. The percentage of mino tautomer is estimated as $10 \pm 2\%$ and its mean life time is about 10^{-3} sec. in the neutral aqueous solution.

APPENDIX I

The general three sites exchange problem:

$$C \underset{\text{fast}}{\rightleftharpoons} A \underset{\text{slow}}{\rightleftharpoons} B$$

Assuming that the exchange between C and A is very fast, while the reaction between A and B is relatively slow and there is no direct exchange between C and B, we may set the Bloch equations modified by the effect of chemical exchange.[12]

$$\frac{dG_A}{dt} + \alpha_A G_A = -i\omega_1 M_o^a + \frac{G_B}{\tau_b} + \frac{G_C}{\tau_c} \tag{1}$$

$$\frac{dG_B}{dt} + \alpha_B G_B = -i\omega_1 M_o^b + \frac{G_A}{\tau_{ab}} \tag{2}$$

$$\frac{dG_C}{dt} + \alpha_C G_C = -i\omega_1 M_o^b + \frac{G_A}{\tau_{ac}} \tag{3}$$

where G_A, G_B, and G_C represent the complex magnetic moments of these three species respectively, and $\alpha_A = \frac{1}{T_{2A}} + \frac{1}{\tau_{ab}} + \frac{1}{\tau_{ac}} - i\Delta\omega_A$,

$\alpha_B = \frac{1}{T_{2B}} + \frac{1}{\tau_b} - i\Delta\omega_B$, $\alpha_C = \frac{1}{T_{2C}} + \frac{1}{\tau_c} - i\Delta\omega_C$,

T_{2A}, T_{2B} and T_{2C} denote their natural transverse relaxation time in the absence of exchange.

τ_{ab}, τ_{ac}, τ_b and τ_c are defined as in the previous section.

ie; τ_a, τ_b and τ_c are their mean life time respectively,

$$G_B = \frac{i\dot{\omega}_1 \left[\overset{a}{M_0} \left(\frac{\alpha_C}{\tau_{ab}} \right) + \overset{b}{M_0} \left(\alpha_A \alpha_C - \frac{1}{\tau_{ac}\tau_c} \right) + \overset{c}{M_0} \left(\frac{1}{\tau_c \tau_{ab}} \right) \right]}{- \alpha_A \alpha_B \alpha_C + \frac{\alpha_B}{\tau_{ac}\tau_c} + \frac{\alpha_C}{\tau_{ab}\tau_b}} = U_B + iV_B \qquad (8)$$

$$G_C = \frac{i\dot{\omega}_1 \left[\overset{a}{M_0} \left(\frac{\alpha_B}{\tau_{ac}} \right) + \overset{b}{M_0} \left(\frac{1}{\tau_{ac}\tau_b} \right) + \overset{c}{M_0} \left(\alpha_A \alpha_B - \frac{1}{\tau_{ab}\tau_b} \right) \right]}{- \alpha_A \alpha_B \alpha_C + \frac{\alpha_B}{\tau_{ac}\tau_c} + \frac{\alpha_C}{\tau_{ab}\tau_b}} = U_C + iV_C \qquad (9)$$

where $\overset{a}{M_0} = P_A M_0$; $\overset{b}{M_0} = P_B M_0$; $\overset{c}{M_0} = P_C M_0$; and P_A, P_B, and P_C are their relative populations.

Since G_A, G_B, and G_C are complex quantity, we can express them respectively in terms of real part and imaginary part; the former is referred to as the in phase or dispersion moment (U_A, U_B, U_C), and the latter is the out-of phase or absorption moment (V_A, V_B, V_C).

If we assume that all the exchanges involved are in the fast exchange limit, ie; $\tau_a^{-1} \gg \Delta\dot{\omega}_A \gg T_{2A}^{-1}$; $\tau_b^{-1} \gg \Delta\dot{\omega}_B \gg T_{2B}^{-1}$; $\tau_c^{-1} \gg \Delta\dot{\omega}_C \gg T_{2C}^{-1}$ and also $\tau_c^{-1} \gg \tau_b^{-1}$.

We may simplify Eq. (7), (8), and (9) into the following forms after the complicated calculation and proper approximations.

$$G_A = \frac{\dot{\omega}_1 M_o^a \{ [(P_A + P_C)\, \tau_B\, \Delta\dot{\omega}_B + (P_A + P_B)\, \tau_C\, \Delta\dot{\omega}_C\ ] + i \}}{\{ [\pi \Delta \nu_o + \frac{P_B^2}{P_A} (1 - P_B)^2\, (\dot{\omega}_{AC} - \dot{\omega}_B)^2\, \tau_{ab}\ ] + i\, (\dot{\omega}_o - \dot{\omega}) \}}$$

$$= \frac{\dot{\omega}_1 M_o^a \{ [(P_A + P_C)\, \tau_B\, \Delta\dot{\omega}_B + (P_A + P_B)\, \tau_C\, \Delta\dot{\omega}_C\ ] + i \}}{\{ (\frac{1}{T_2})_{obs} + i\, (\dot{\omega}_o - \dot{\omega}) \}} \qquad (10)$$

where $\Delta\nu_o = \frac{P_A}{T_{2A}} + \frac{P_B}{T_{2B}} + \frac{P_C}{T_{2C}}$ is the observed linewidth in the absence of exchange.

$\dot{\omega}_o = P_A \dot{\omega}_A + P_B \dot{\omega}_B + P_C \dot{\omega}_C$ is the observed resonant frequency.

$\dot{\omega}_{AC} = \frac{P_A \dot{\omega}_A + P_C \dot{\omega}_C}{P_A + P_C}$ is the average resonant frequency between species A and C.

$(T_2)_{obs}$ is the observed transerve relaxation time where

$$(\frac{1}{T_2})_{obs} = \pi \nu_o + \frac{P_B^2}{P_A} (1 - P_B)^2\, (\dot{\omega}_{AC} - \dot{\omega}_B)^2\, \tau_{ab} \qquad (11)$$

represents the observed linewidth.

The component of the complex moment which is important to our NMR study is its imaginary one (V_A, V_B, V_C) which can be obtained after the rationalization of the complex moment.

$$V_A = \frac{\dot{\omega}_1 M_o^a \left[\left(\frac{1}{T_2}\right)_{obs} - (\dot{\omega}_o - \dot{\omega}) \, (P_A + P_C) \, \tau_B \, \Delta\dot{\omega}_B \right]}{(\dot{\omega}_o - \dot{\omega})^2 + \left[\left(\frac{1}{T_2}\right)_{obs} \right]^2} \qquad (12)$$

Now absorption moment V_A is simplified as a standard form of Lorentz line shape. By similar mathematical treatment we can get G_B and G_C from which we can calculate V_B and V_C, ie;

$$V_B = \frac{\dot{\omega}_1 M_o^b \left[\left(\frac{1}{T_2}\right)_{obs} - (\dot{\omega}_o - \dot{\omega}) \, \tau_b \, (P_A \, \Delta\dot{\omega}_A + P_C \, \Delta\dot{\omega}_C) \right]}{(\dot{\omega}_o - \dot{\omega})^2 + \left[\left(\frac{1}{T_2}\right)_{obs} \right]^2} \qquad (13)$$

$$V_C = \frac{\dot{\omega}_1 M_o^c \left[\left(\frac{1}{T_2}\right)_{obs} - (\dot{\omega}_o - \dot{\omega}) \, \tau_b \, \Delta\dot{\omega}_B \, (P_A + P_C) \right]}{(\dot{\omega}_o - \dot{\omega})^2 + \left[\left(\frac{1}{T_2}\right)_{obs} \right]^2} \qquad (14)$$

If the exchanges are fast, only one resonance will appear in $\dot{\omega}_o$ which would be properly represented by $V_{obs} = V_A + V_B + V_C$ in Eq. (15).

$$V_{obs} = \frac{\dot{\omega}_1 M_o \left\{ \left(\frac{1}{T_2}\right)_{obs} - (\dot{\omega}_o - \dot{\omega}) \, \tau_b \, (P_A + P_C) \left[(P_A + P_C) \, \Delta\dot{\omega}_B + P_B \, \Delta\dot{\omega}_{AC} \right] \right\}}{(\dot{\omega}_o - \dot{\omega})^2 + \left[\left(\frac{1}{T_2}\right)_{obs} \right]^2} \qquad (15$$

When the exchange rate between A and B is slow, ie; $\tau_B^{-1} \ll \Delta\dot{\omega}_B$, but that between A and C still remains fast, then the

resonance of B will be separated from that of A and C. By approximation, we may calculate the complex moment and relaxation time of each resonance.

$$G_A = \frac{\dot{\omega}_1 M_o^a \left[(P_A + P_C)\,\Delta\dot{\omega}_B + i\,\tau_B^{-1} \right]}{\left[(\dot{\omega}_{AC} - \dot{\omega}) + i(\frac{1}{T_2})_{AC}\right]\left[(\dot{\omega}_B - \dot{\omega}) + i(\frac{1}{T_2})_B\right]} \tag{16}$$

$$G_B = \frac{\dot{\omega}_1 M_o^b \left[(P_A + P_B)\,\Delta\dot{\omega}_{AC} + i\,\tau_B^{-1} \right]}{\left[(\dot{\omega}_{AC} - \dot{\omega}) + i(\frac{1}{T_2})_{AC}\right]\left[(\dot{\omega}_B - \dot{\omega}) + i(\frac{1}{T_2})_B\right]} \tag{17}$$

$$G_C = \frac{\dot{\omega}_1 M_o^c \left[(P_A + P_C)\,\Delta\dot{\omega}_B + i\,\tau_B^{-1} \right]}{\left[(\dot{\omega}_{AC} - \dot{\omega}) + i(\frac{1}{T_2})_{AC}\right]\left[(\dot{\omega}_B - \dot{\omega}) + i(\frac{1}{T_2})_B\right]} \tag{18}$$

where

$$(\frac{1}{T_2})_{AC} = \pi\,\Delta\nu_{oAC} + P\!\!\!/_B\,\tau_b^{-1} = \pi\,\Delta\nu_{oAC} + P\!\!\!/_A\,\tau_{ab}^{-1} \tag{19}$$

$$(\frac{1}{T_2})_B = \pi\,\Delta\nu_{oB} + (P\!\!\!/_A + P\!\!\!/_C)\,\tau_b^{-1} \tag{20}$$

where $\pi\,\Delta\nu_{oAC} = \frac{1}{P_A + P_C}\left(\frac{P_A}{T_{2A}} + \frac{P_C}{T_{2C}}\right)$ and $\pi\,\Delta\nu_{oB} = \frac{1}{T_{2B}}$ represent the natural linewidth. $(T_2)_{AC}$ is the relaxation time for the resonance AC and $(T_2)_B$ is that for the resonance B. Thus $(\frac{1}{T_2})_{AC}$.

and $(\frac{1}{T_2})_B$ represent their respective linewidths. By suitable treatment, we may separate the total moment $G = G_A + G_B + G_C$ into two terms which represent the moment of the resonances of AC and B respectively. Its imaginary part can also be separated into two terms, ie;

$$V_{obs} = V_A + V_B + V_C = \frac{\overline{X}}{(\dot{\omega}_{AC} - \dot{\omega})^2 + [(\frac{1}{T_2})_{AC}]^2} + \frac{\overline{Y}}{(\dot{\omega}_B - \dot{\omega})^2 + [(\frac{1}{T_2})_B]^2}$$

where $\overline{X}$ and $\overline{Y}$ are complicated functions for these parameters.

From the absorption moment V_{obs}, we might understand that there are two resonances which occur at frequency $\dot{\omega}_{AC}$ and $\dot{\omega}_B$ when the exchange rate between A and B is slow compared to their chemical shift difference.

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FIGURE CAPTIONS

Fig. 1. Abnormal A-C Pair and Normal G-C Pair.

Fig. 2. pD Dependence of Chemical Shift of H_5 Resonances of Cytosine and Related Compounds.

Fig. 3. pD Dependence of Chemical Shift of H_6 Resonances of Cytosine and Related Compounds.

Fig. 4. pD Dependence of Linewidth of H_5 Resonances of Cytosine and Related Compounds.

Fig. 5. Spectra of H_5 Resonance of 5′ CMP at Different pDs.

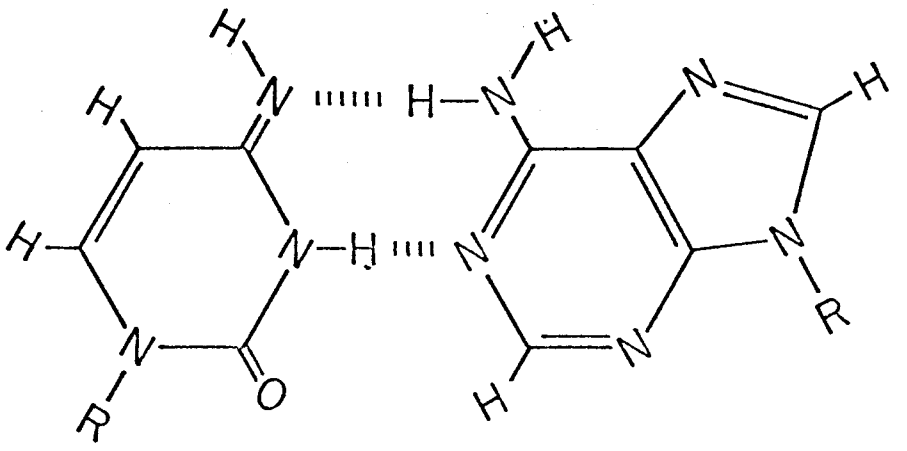
Fig. 6. Temperature Study of H_5 Resonance of 5′ CMP at Different pDs.

Fig. 7. Spectra of H_5 Resonance of 5′ CMP at Different Temperatures.

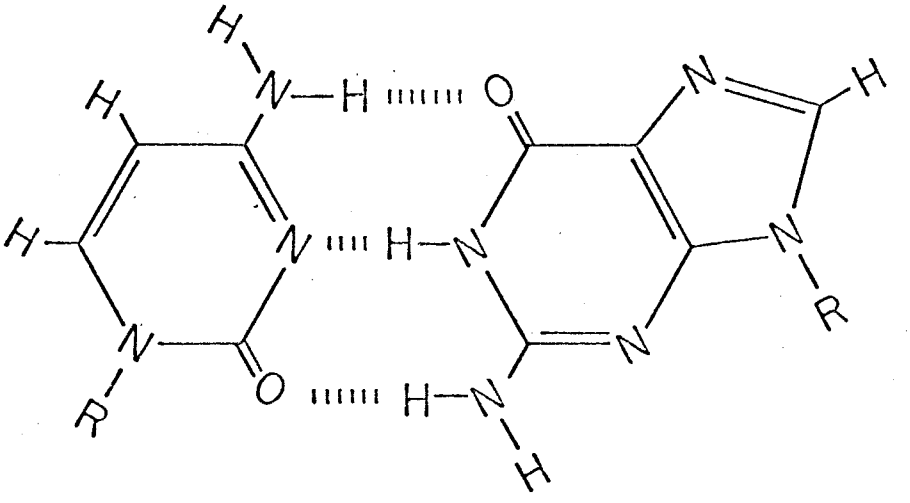
Fig. 8. Field Dependence Study of H_5 Resonance of 5′ CMP at 16°C and pD 5.0.

Fig. 9. Computer Simulation of pD Dependence of Linewidth of H_5 Resonance of 5′ GMP.

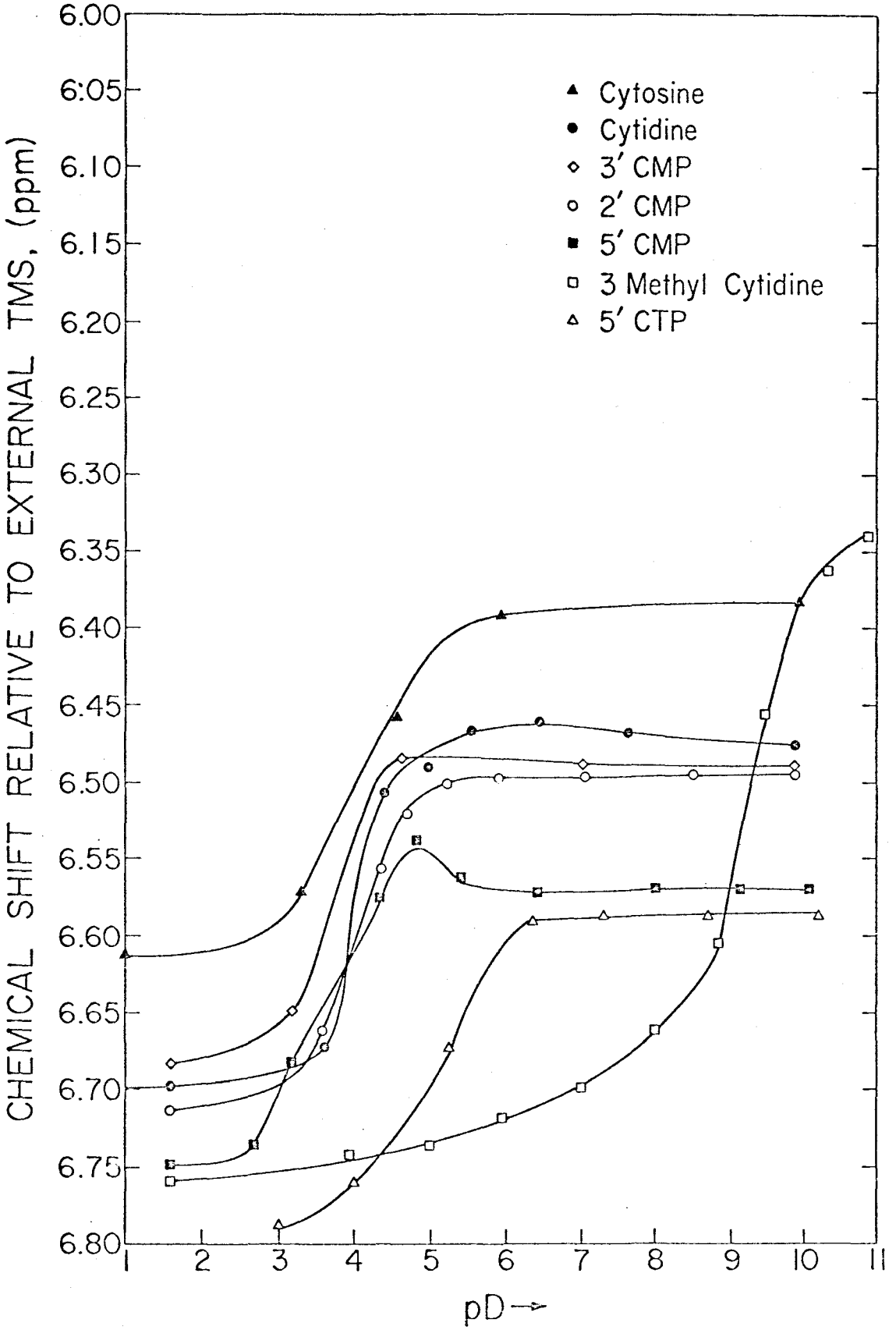
Fig.10. Computed Spectrum of H_5 Resonance of 5′ CTP at -20°C and pD 5.6.

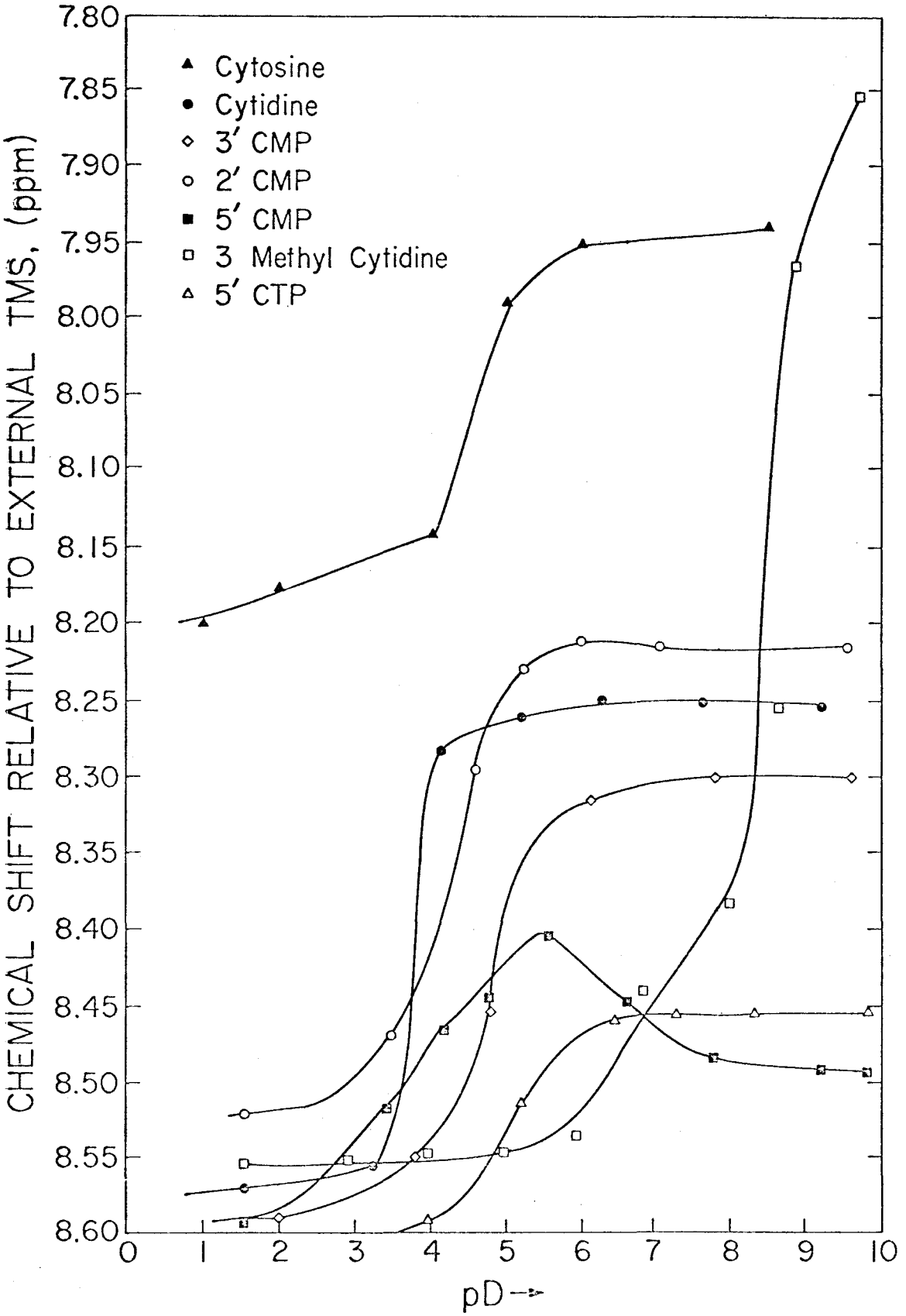


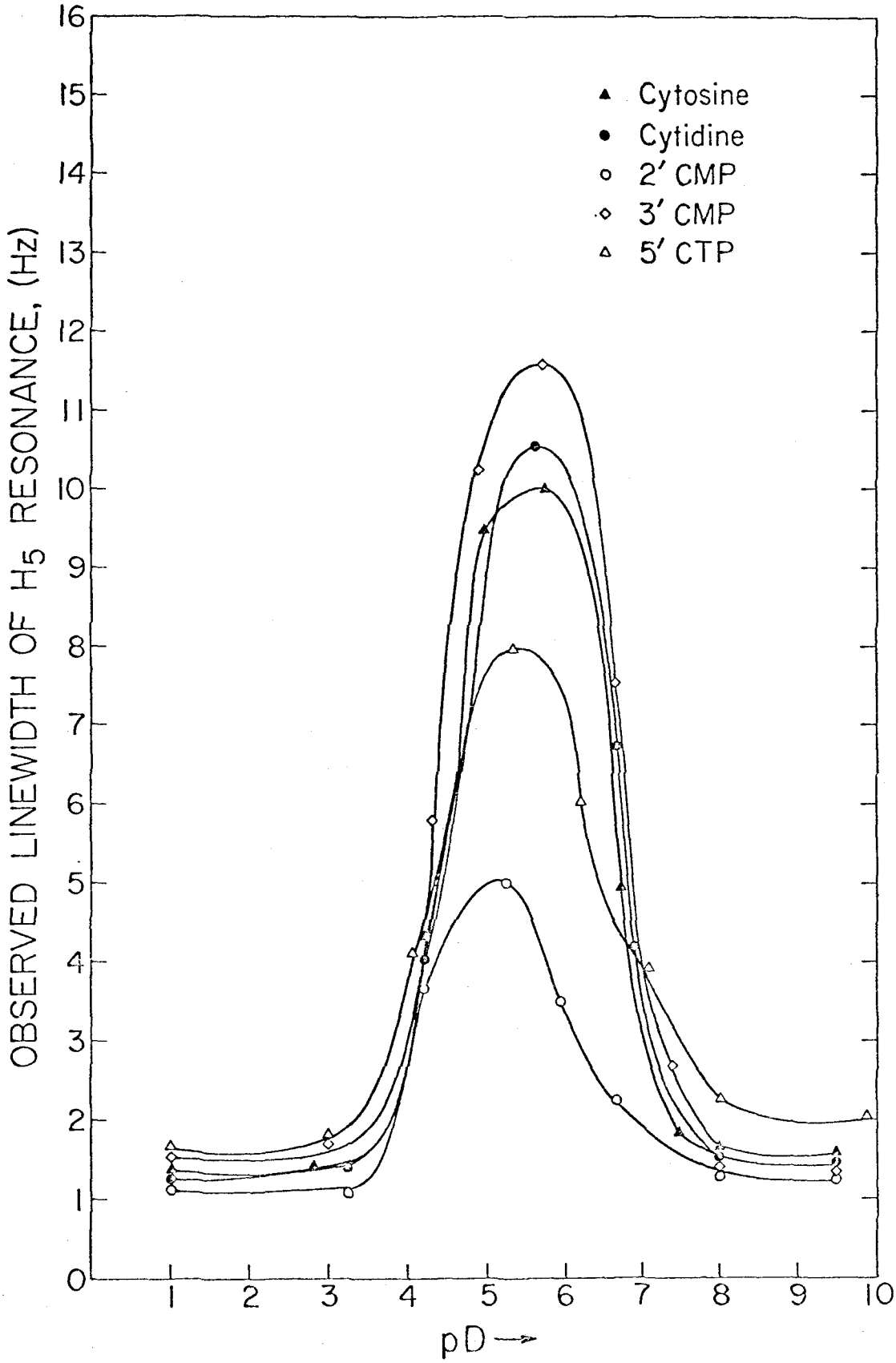
C — A

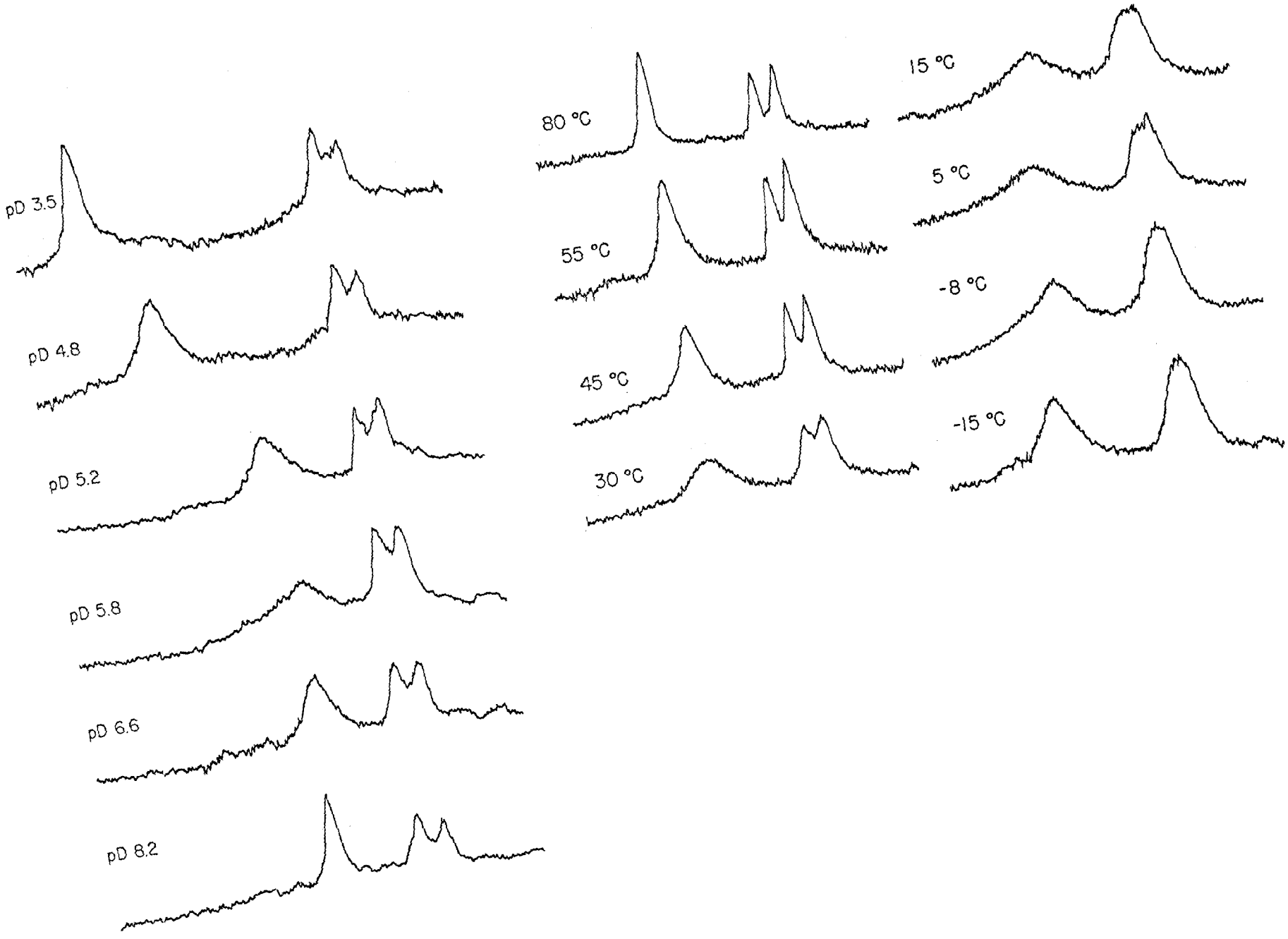


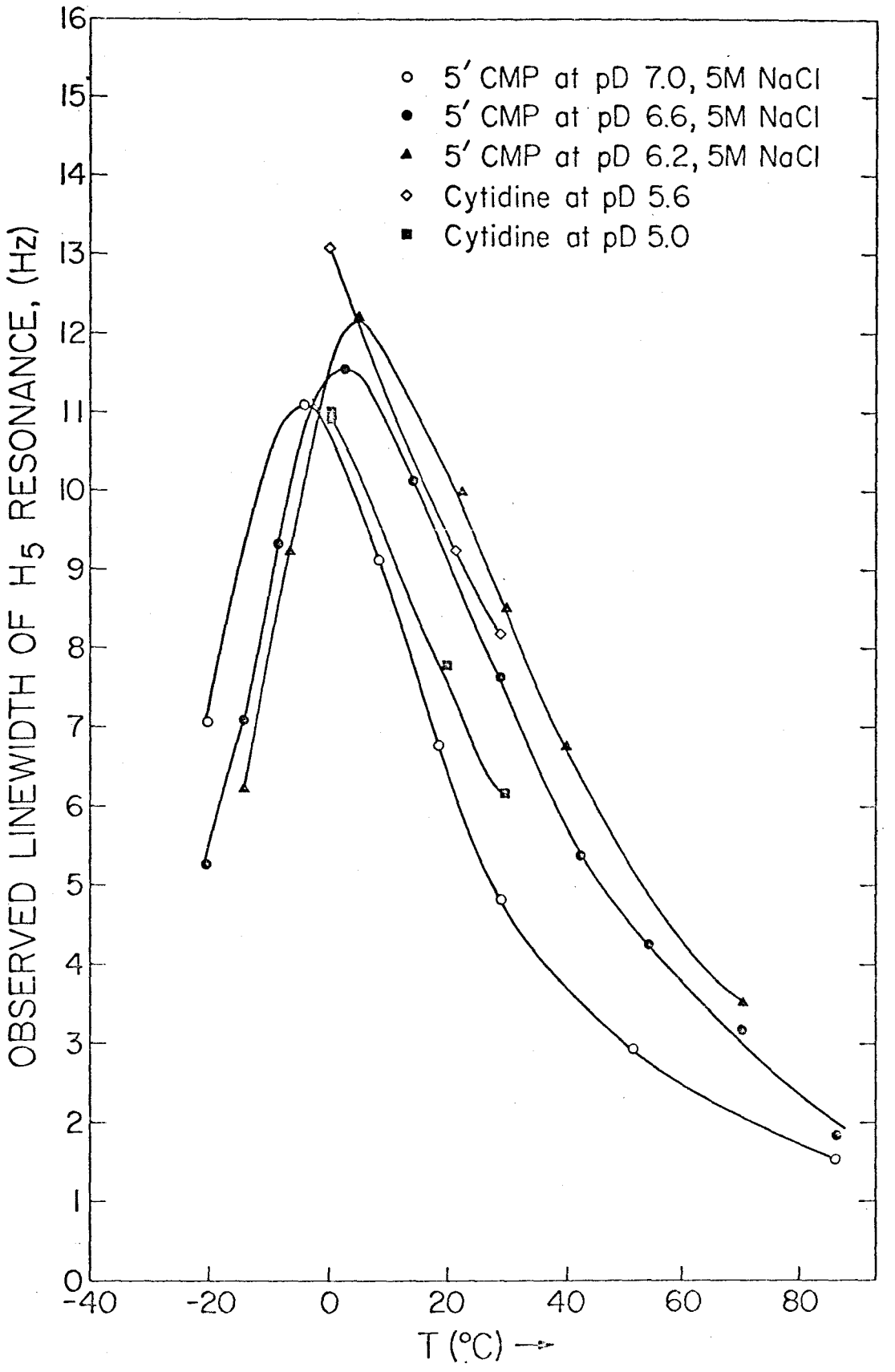
C — G

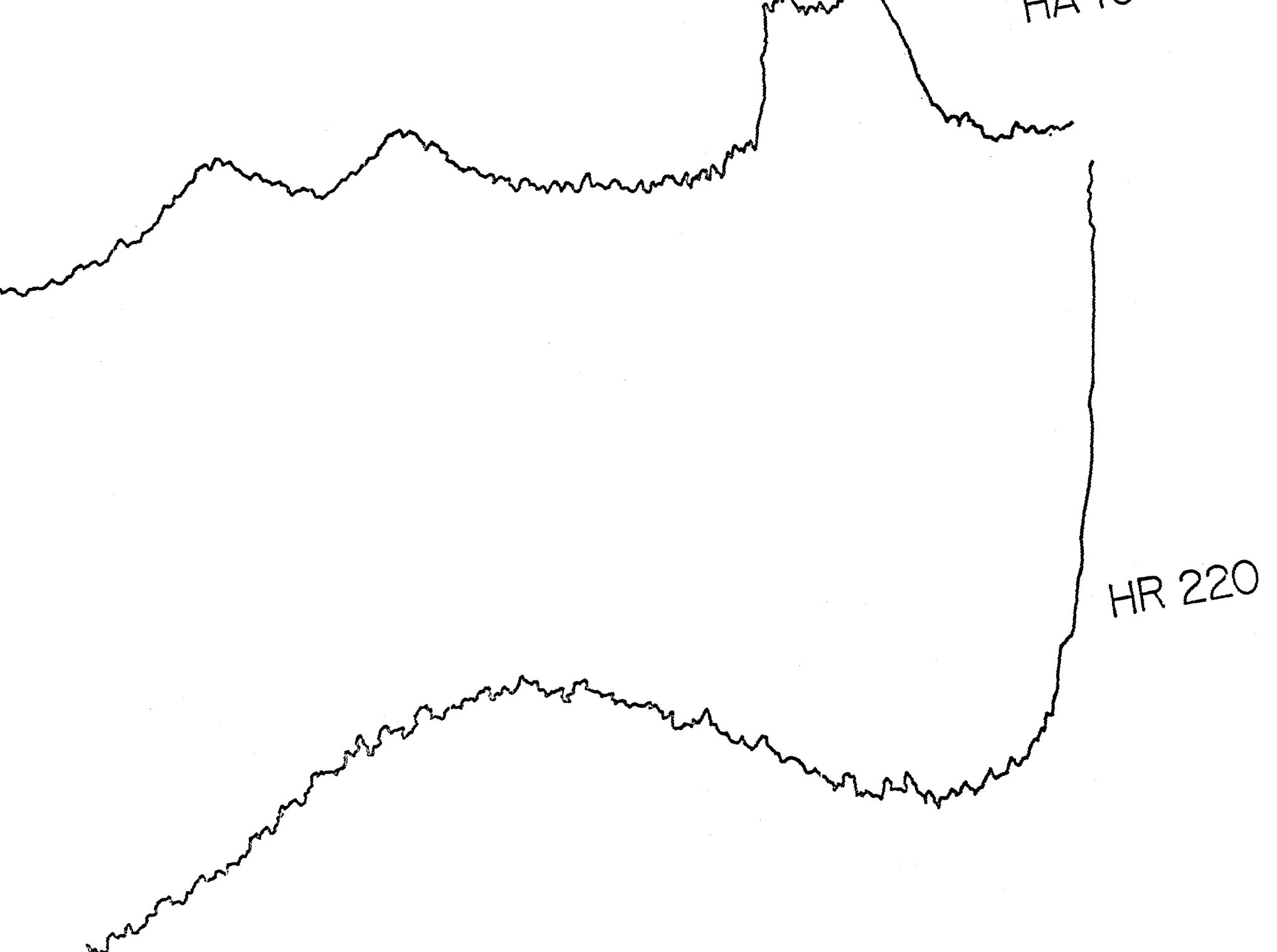

CHEMICAL SHIFT RELATIVE TO EXTERNAL TMS, (ppm)
6.00
6:05
6.10
6.15
6.20
6.25
6.30
6.35
6.40
6.45
6.50
6.55
6.60
6.65
6.70
6.75
6.80
1
2
3
4
5
6
7
8
9
10
11
pD→
Cytosine
Cytidine
3′ CMP
2′ CMP
5′ CMP
3 Methyl Cytidine
5′ CTP

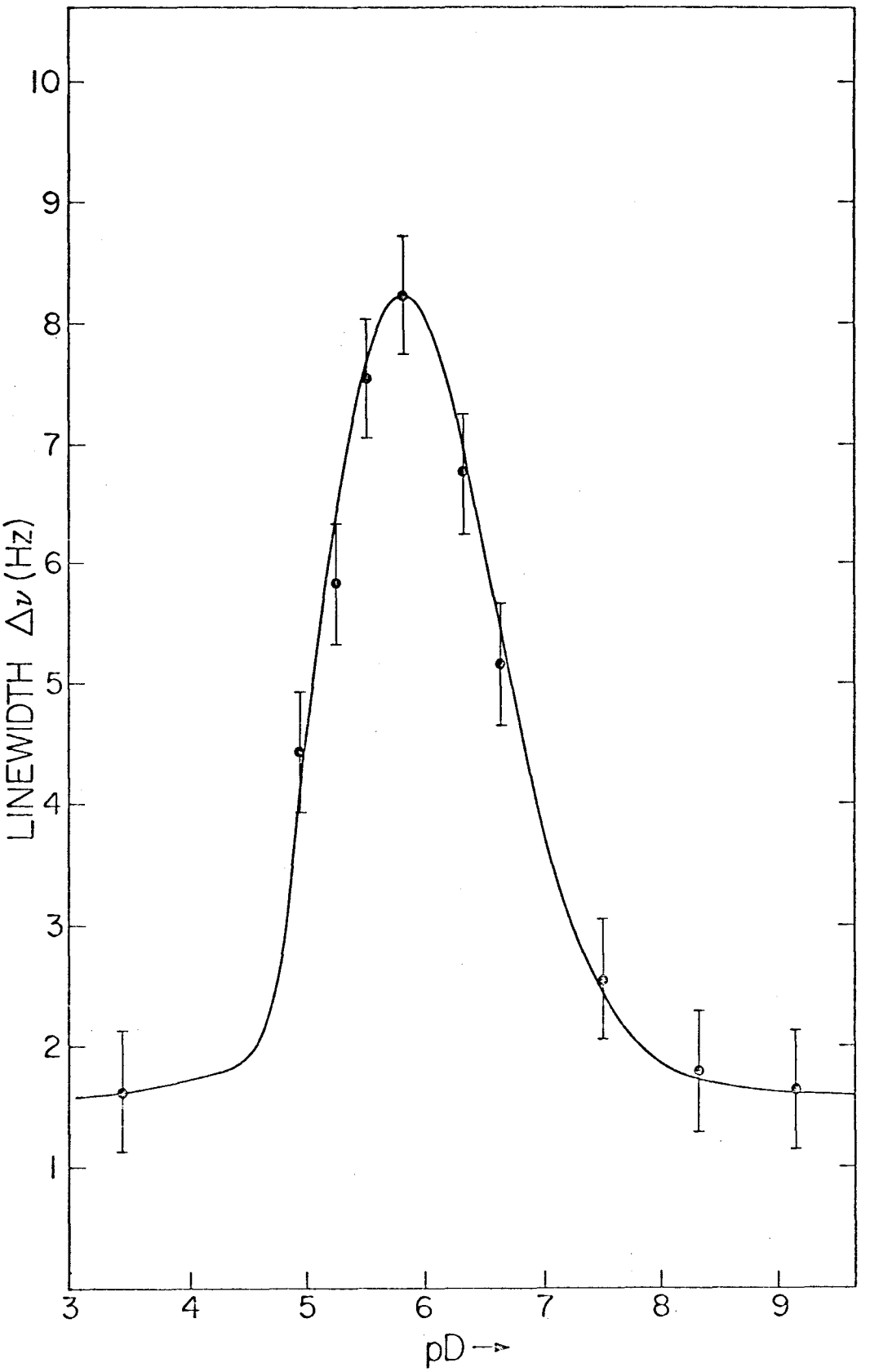

Cytosine
Cytidine
3′ CMP
2′ CMP
5′ CMP
3 Methyl Cytidine
5′ CTP
CHEMICAL SHIFT RELATIVE TO EXTERNAL TMS, (ppm)
7.80
7.85
7.90
7.95
8.00
8.05
8.10
8.15
8.20
8.25
8.30
8.35
8.40
8.45
8.50
8.55
8.60
0
1
2
3
4
5
6
7
8
9
10
pD →

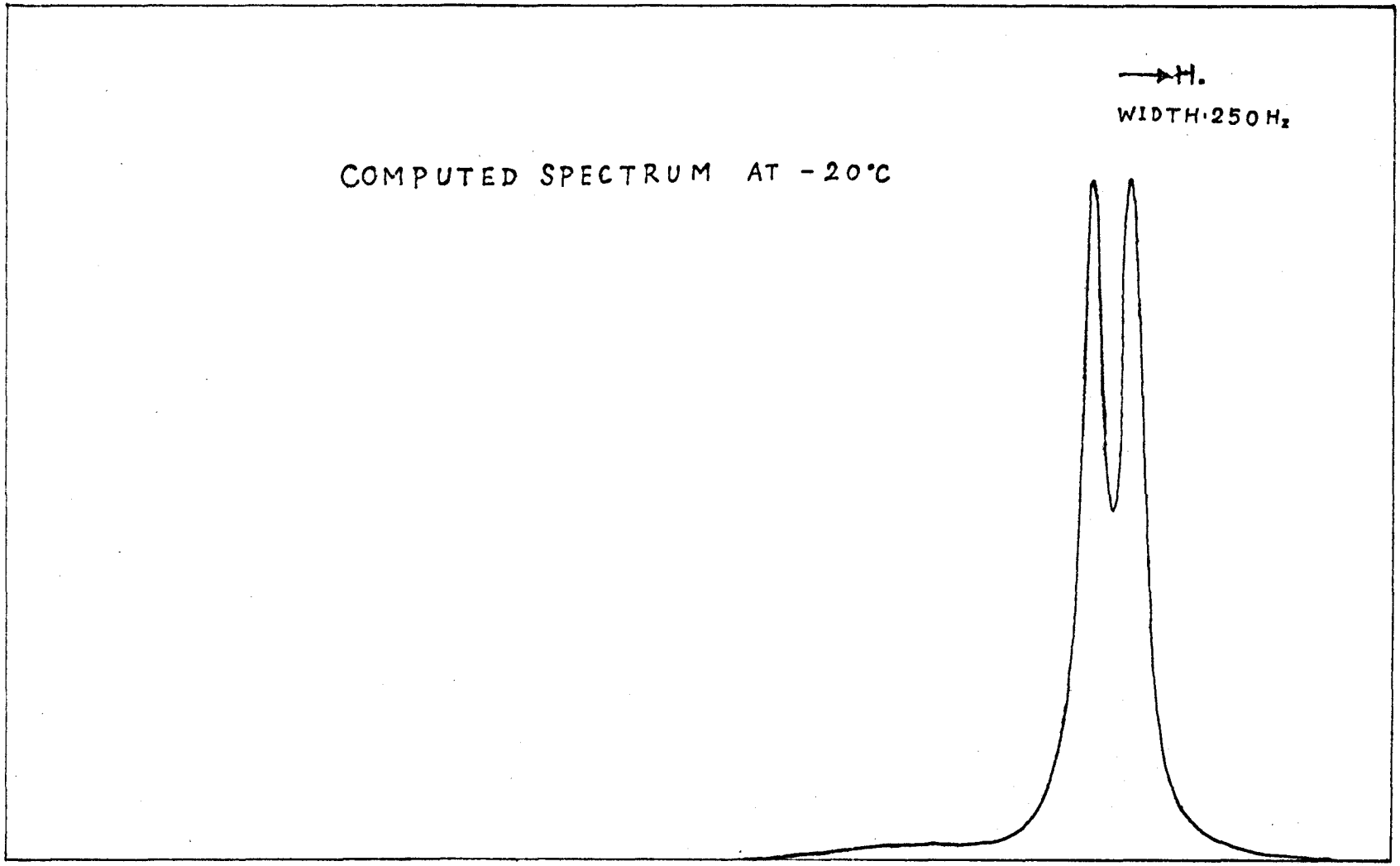

OBSERVED LINEWIDTH OF H_5 RESONANCE, (Hz)
pD →
Cytosine
Cytidine
2′ CMP
3′ CMP
5′ CTP
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
1
2
3
4
5
6
7
8
9
10


pD 3.5
pD 4.8
pD 5.2
pD 5.8
pD 6.6
pD 8.2
80 °C
55 °C
45 °C
30 °C
15 °C
5 °C
-8 °C
-15 °C


5′ CMP at pD 7.0, 5M NaCl
5′ CMP at pD 6.6, 5M NaCl
5′ CMP at pD 6.2, 5M NaCl
Cytidine at pD 5.6
Cytidine at pD 5.0
OBSERVED LINEWIDTH OF H5 RESONANCE, (Hz)
T (°C) →
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
-40
-20
0
20
40
60
80


HR 220


LINEWIDTH Δν (Hz)
10
9
8
7
6
5
4
3
2
1
3
4
5
6
7
8
9
pD →


COMPUTED SPECTRUM AT -20°C
H.
WIDTH·250 Hz

The Tautomeric Exchange of Nucleic Acids (II)

Abstract

From the PMR study of guanosine and its related compounds at different pD, temperature and concentrations, it was observed that H_8 resonances of guanine base in some of these compounds are unusually broad over a wide pD range (from 2 to 8) in aqueous solutions. This phenomenon is explained in terms of the effects of base pairing, stacking and tautomeric exchange between the 1 and 6 positions of guanine base. The observation of sharp resonance of H_8 of 1-methyl guanosine having only one possible tautomeric structure supports that there exists minor tautomer of this base. This tautomeric exchange is catalyzed either by solvent (D_2O), base (OD^-) or pairing of base. The percentage of minor tautomer and the activation energy of the exchange are also estimated from the kinetic analysis.

Introduction

The behavior of guanosine and its related compounds in aqueous solution at different pH and temperature has been studied by Gellert,[1, 2] et al either by UV, IR, optical rotation or X-ray crystalgraphy. It has been observed that at intermediate pH and relatively concentrated solution, guanosine, 3′ GMP, and 5′ GMP form very viscous gel at room temperature, while there is little tendency for 2′ GMP to form gel. This would indicate that there are strong pairing and stacking

effects between guanine bases in some of these compounds. From X-ray crystalgraphical study, we know that these gel forming compounds will aggregate to form the tetramer first and then stack vertically each other leading to the formation of a regular helical structure. Similarly, from our NMR study, we have observed that the H_8 resonance of guanine base in these compounds has shown irregular broadening over a wide pD and temperature range. Although this phenomenon would be more or less related to the formation of viscous gel, evidences presented in our study strongly suggest that there is tautomeric exchange between 1 and 6 positions of guanine base which also contributes to the line broadening of H_8 resonances. From the analysis of the linewidth and chemical shift of H_8 resonances of these compounds under different experimental conditions it is possible to resolve various complicated effects from which may deduce that the minor enol tautomer exists for this base.

Experimental Section

1. Instrumentation.

NMR spectra were recorded with Varian Associates HA-100 spectrometer with the aid of C1024 time average computer to enhance the signal to noise ratio. HR-220 spectrometer was used for the field dependence study. All the spectra were recorded with constant temperature probe except those for temperature study for which a variable temperature probe was used.

The probe temperature is usually kept at 30 ± 1 °C. Chemical

shift was measured relative to external TMS capillary using a lock system in HA-100 spectrometer. The accuracy of this measurement is ± 1 H_z. No magnetic susceptibility correction was made for all study.

The Ostwald viscosmeter was used to measure the relative viscosity of sample solution in thermostat where the temperature was kept at 32 ± 0.1°C.

Ultraviolet absorption was measured with a Cary Model 14 spectrophotometer, using two 0.1 mm cells and keeping the temperature at 25 ±1°C.

2. Materials.

The following compounds were studied either in free acid or sodium salt form: 5′GMP, 3′ GMP, 2′ GMP and guanosine which were purchased from either Sigma Chemical Company or Calbiochem Company with grade A reagents.

2-NN′ dimethylaminomethylene guanosine was obtained from Calbiochem. 1-methyl guanosine was purchased from Cyclochemical Corporation at Los Angeles. Although these samples were obtained with very high purity, Dowex-50 Cation Exchanger was used to purify the sample.

All the samples were prepared in 99.7% D_2O solution supplied by Columbia Organic Chemicals, Columbia, South Carolina.

The solution pDs were adjusted with either IN DCI and IN Na OD. A Northrop and Leeds 7401 PH meter with miniature electrodes

was used to measure the pD of the solution which was taken as the observed PH meter reading plus 0.4.

Result

1. Guanosine and 5'GMP.

Since all the other protons are deuterium exchanged except H_8, this is the only resonance which we can study on guanine base. The chemical shift behavior of H_8 resonance of guanosine and its derivatives in aqueous solution at different pDs has been previously reported.[3,4] The large upfield shift of H_8 resonance between pD 2 to 4 indicates that the deprotonation occurs on 7-N position of base as shown in Fig. (1).

As shown in Fig. (2), the linewidth of H_8 resonance is strongly pD dependent. At both very high and low pDs, it approaches the natural linewidth limit, but becomes broad at intermediate pD region with maximum width 10 and 6 H_z at pDs around 3.5 and 6.5 respectively and also with minimum width from 3.5 to 5 H_z at pD~5.0 depending on the compounds and concentration studied.

Because of the limitation of solubility, the concentration used in guanosine study is only 5×10^{-3} M and a little turbility was observed at intermediate pDs.

The linewidth of H_8 resonance of 5'GMP is also concentration dependent as shown in Fig. (2), especially at low concentration and intermediate pD regions, while its chemical shift doesn't change obviously with concentration. Its linewidth changes from 3 to 6 H_z

as the concentration varies from 10^{-1} to 3×10^{-3} M. It was observed that 5'GMP forms very viscous gel at higher concentrations ($> 5 \times 10^{-2}$ M) at room temperature.

2. 2'GMP, 3' GMP, 2NN' dimethyaminomethylene-guanosine and 5' GTP.

The chemical shift behavior of H_8 resonance for these compounds at different pDs is roughly the same as that of guanosine; but its linewidth behavior is somewhat different. Instead of having a minimum linewidth at pD ~ 5.0, it is the broadest among the whole pD region varying from 15 to 20 H_z depending on the compounds and concentration, while it is gradually sharp at both higher and lower pD.

As shown in Fig. (5), the linewidth is also concentration dependent at intermediate pD, when the concentration changes from 0.1 M to 0.01 M the linewidth varies from 18 to 10 H_z for 2'GMP and 3' GMP. 3'GMP solution is also viscous at higher concentration (10^{-1} M) and intermediate pD region at room temperature, while 2' GMP solution is not. At pD higher than 7, its linewidth is shown to be concentration independent. The H_8 resonance of 5' GTP is also broad at the middle pDs with maximum width 6.6 H_z at pD ~ 5.0.

3. Viscosity Study.

The relative viscosity of GMP isomers at different pDs was measured at 32 ± 0.1 °C with the Ostwald viscosmeter. The concentration studied was 0.025 M and pure water was taken as reference.

The result of this study is given in Fig. (7). From this

measurement, it is easy to see that 5'GMP not only has higher viscosity than any other isomers, but also has its maximum at pD 5.0 which the 2' and 3'GMP do not have.

4. For all the compounds, there is significant change in coupling constant for $H_{1'}$ and $H_{2'}$ protons on ribose. At low pD the coupling constant $J_{H_{1'}-H_{2'}}$ is 3.8 H_z. As pD is higher the $J_{H_{1'}-H_{2'}}$ increases gradually up to 6.2 H_z at pD $>$ 7.0. Fig. (9)

5. Field Dependence Study.

Under the same condition, the linewidth of H_8 resonance measured by both HA-100 and HR-220 spectrometer is different. For a solution containing 0.02 M of 2' GMP at pD 7.5 and 30°C, the H_8 linewidth measured by HA-100 is 3.8 H_z while those measured by HR-220 is 5.9 H_z.

6. Temperature Study.

The temperature contours of the linewidth of H_8 resonances of 5' GMP and 2' GMP at different pDs are shown in Figs. (2) and (3).

For 5'GMP, the temperature study of H_8 resonance shows that it is sharp at higher temperature and broad at lower temperature for the whole pD range. At pD 5.0 and 20°C, there is a melting transition which can be observed from the large discontinuous change in linewidth about this temperature. For 2' and 3'GMP the same melting transition was also observed, but it seems to occur at lower temperature.

At higher pD (> 7.0), no transition can be observed; instead it is sharp again below certain temperature. Under high salt concentration, there is no transition behavior for 2′ GMP which can be detected from the changes in linewidth at low temperature.

7. UV Study.

The pD and concentration dependence of the optical density at absorption maximum for 5′ GMP is shown in Fig. (6). It is characterized that there is maximum absorbance at pD 5.0 and concentration higher than 5×10^{-3} M at λmax 253 mμ with absorbance at pD 8.0 as reference, while the same phenomenon is not observed for 2′ and 3′ isomers under the same condition.

8. We also did the same pD dependence study of 1-methylguanosine on which N-1 position is substituted with methyl group. As a result we observed only sharp H_8 resonance for all pD and concentration range.

Discussion

From our PMR study of H_8 resonances of these compounds, and from the results of UV study and viscosity measurement, we may assume that there are several factors which contribute to the transverse relaxation time (T_2) of H_8 resonance of guanine base which is better represented by its observed linewidth.

First, let us consider the possibility of tautomeric exchange occuring on guanine base between 1 and 6 or 2 and 3 positions. Since

the H_8 proton is moving between different tautomeric environments due to exchange, its transverse relaxation time will be reduced to certain extent depending on the rate of exchange. Because the observed linewidth is pD dependent, we may predict that the chemical exchange is possibly catalyzed by the solvent (D_2O) or its ionized species (D^+, OD^-).

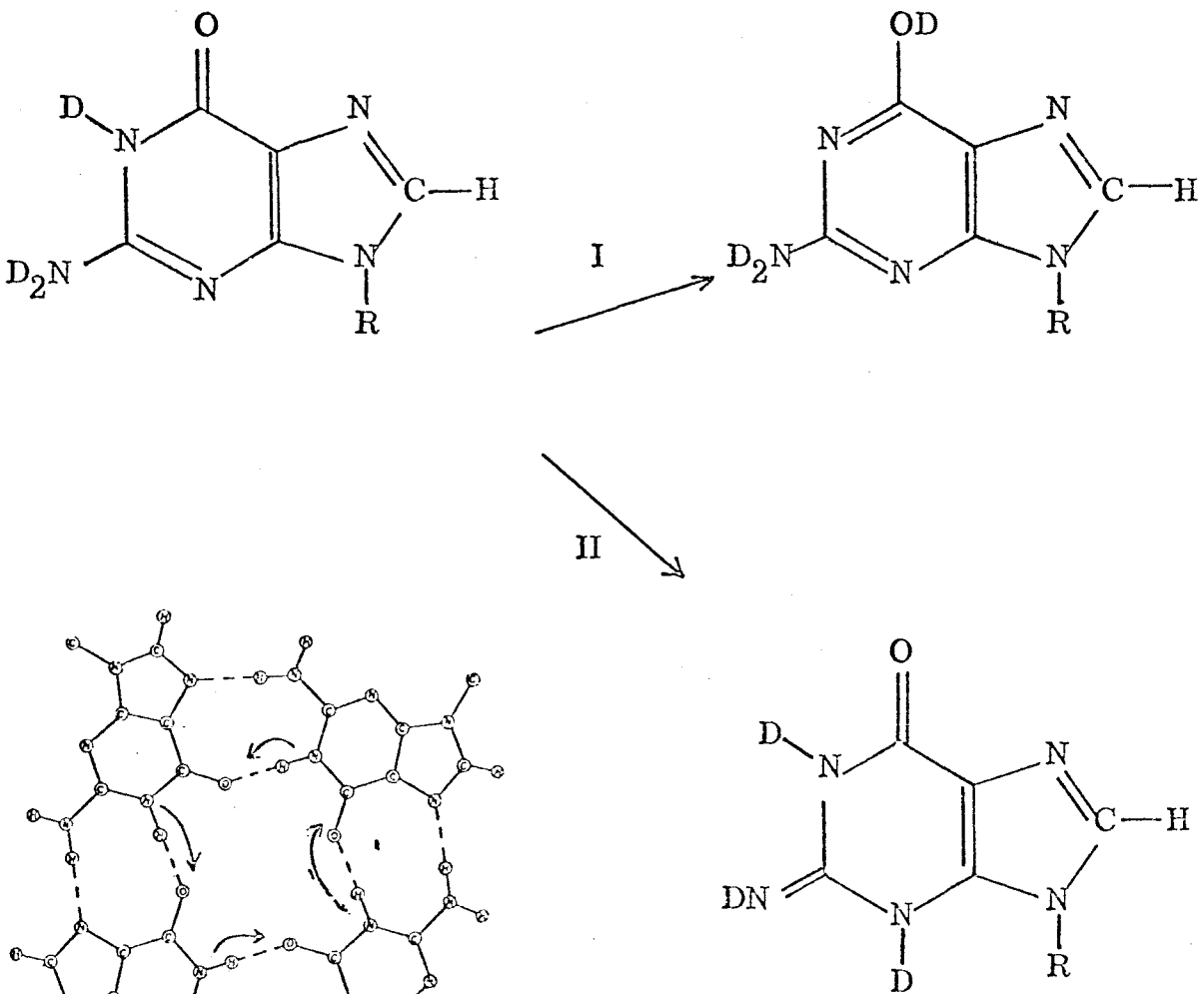


FIG. 10--Proposed arrangement of the bases in GMP gels.

Molecular association is also a chemical exchange process and would contribute to line broadening of H_8 resonance, if its exchange rate is on the NMR time scale. From previous study by optical rotation, UV and X-ray crystalgraphy it has been observed that there are strong intermolecular associations in aqueous solution for guanosine and some GMP isomers. This association will lead to the formation of tetramers and these are then folded each other vertically by stacking between planar tetramers to form regular helical structure at intermediate pD and at room temperature.

The stability of the molecular association is sensitive to the strength of intermolecular hydrogen bond and hence sensitive to the conformation of the base and ribose and position of phosphate group.

From the viscosity measurement and UV study, we may conclude that there is the strongest intermolecular association for guanosine and 5'GMP; 3'GMP is the next, while there is little tendency for 2'GMP and 5' GTP to form helical structure which would be the most stable at pD 5.0, when the base is neutral and phosphate group is mononegatively charged. At higher concentrations, the equilibrium is in favor of this intermolecular association, for the strong stacking effect will enhance the molecular aggregation and make the solution more viscous.

From our NMR study of H_8 resonance of these compounds under different conditions, it is possible to estimate the rate and degree of this aggregation and tautomerism.

The chemical processes which are involved in the molecular

association and tautomeric exchange may be expressed as the following scheme:

$$\begin{array}{ccc} & \overset{\text{I}}{} & \\ G & \rightleftharpoons & G^{*} \\ \Vert\!\downarrow \text{III} & & \Vert\!\downarrow \\ (G_4)_n & \underset{\text{IV}}{\rightleftharpoons} & G_4 \underset{\text{II}}{\rightleftharpoons} G_4^{*} \end{array}$$

where G is monomeric major tautomer,

G^{*} is monomeric minor tautomer,

G_4 is tetramer,

G_4^{*} is tetramer in its tautomeric form,

$(G_4)_n$ is the aggregated tetramers.

The process I and II are tautomeric exchange reactions.

The process III is overall process for the tetrameric formation.

The process IV is the reaction to form the helical structure by vertical stacking.

The experimental results have shown that H_8 resonances of these compounds are very sensitive to solution pD and temperature which are deeply related to the rate and equilibrium of these processes. The maximum relative absorbance and viscosity for 5′GMP at pD 5.0 would imply that there is high degree of molecular association for this compound. From our PMR study of this compound, it is characterized that there is maximum width of H_8 resonance for guanosine and 5′ GMP at pD ~ 5.0 at higher temperature ($\geq 30°C$), but there is discontinuous change in linewidth about 20°C. The latter would indicate the occurrence

of phase transition near this temperature. Although the same transition phenomenon was observed for 2′ and 3′ isomers, the linewidth behavior is different. This would show us that the rate and equilibrium for these processes are different for each case. If the line broadening of H_8 is from chemical exchange the temperature study would show us that the average rate of all processes is in the fast exchange limit. Since the solution is in multiple step equilibrium, the concentration dependence of linewidth would indicate that the rate and relative weight of each process will change with concentration. From the reaction scheme, we may realize that the percentage of monomer will increase with decrease in concentration and the process for monomeric tautomeric exchange (I) would predominate at very dilute solution. Because there is no obvious change in chemical shift, when the concentration of sample solution varies from 0.5 to 0.003 M, we may assume that the polymerization process (III and IV) are fast relative to the changes in chemical shift due to molecular association and would not give any significant effect on the observed linewidth. While another proton transfer process (II) which will change the tetramer into its tautomeric form would be important to our linewidth study.

Based on the above assumption, the average life time for the overall processes can be expressed as the weight average of the tautomeric exchange processes I and II, ie;

$$(\Delta\nu_{obs} - \Delta\nu_o)^{-1} \quad \alpha \quad \tau_{ave}^{-1} = \frac{P_G \tau_G^{-1} + P_{G_4} \tau_{G_4}^{-1}}{(P_G + P_{G_4})} \qquad (1)$$

where P_G and P_{G_4} are relative population of monomer and tetramer in keto form respectively. Here we assume that τ_G^{-1} and $\tau_{G_4}^{-1}$ are concentration independent at the same pD, because they are unimolecular processes. The observed linewidth can be simply correlated to the exchanging life time by Eq. (1). Since the relative population of monomer and tetramer is concentration dependent, the linewidth would also change with concentration. So the concentration study of the H_8 resonance would give us some information about the rate and equilibrium for each process. From the assumption, we may conclude that the observed transverse relaxation time (T_2) of H_8 resonance is predominately determined by the two tautomeric exchange processes (I and II), but not molecular association. At pD 5.0, by observing the relatively broad H_8 resonance of 2′ and 3′ isomers, we may realize that process (II) is slower than that in 5′GMP.

At high pD (> 7.0), the H_8 linewidth of 2′ GMP is concentration independent. This would indicate that at high pD the equilibrium is shifted to the unimolecular exchange, ie; the monomeric tautomeric exchange predominates because tetramer is relatively unstable at this pD region and monomer exists exclusively.

Temperature study also gives us some information about the dynamic behavior of these chemical reactions. Since the observed linewidth decreases with increase in temperature above 30°C, we know that all the exchange processes are in the fast exchange limit for this temperature range. A discontinuous change in linewidth

near 20°C shows an order-disorder transition which would indicate that high degree of molecular association will increase the correlation time of molecular motion, and thus make the observed resonance broad.

At intermediate pD region, the factors contributing to the observed linewidth are very complicated, but at high (> 7.0) or low pD (> 3.0) region the situation is relatively simple, since the molecular association is weak and the tetramer does not stably exist, the major contribution to the H_8 linewidth would be only from that of the monomeric tautomeric exchange.

Temperature study of 2′ GMP between -20° and 80°C at extreme pD region shows that the linewidth behavior of H_8 resonance is characteristic of chemical exchange. For 0.02 M of 2′ GMP at pD 7.0, the H_8 resonance becomes sharper at temperature higher than 30°C, but broader at lower temperature. At temperature below 10°C, it becomes sharp with decrease in temperature and the maximum linewidth observed is about 9 H_z at 16°C. There are two tautomeric exchanges which are possible on guanine base, ie; that between position 2 and 3, amino-imino tautomers, and position 1 and 6, keto-enol tautomers. The observation of sharp resonance of 1 methyl guanosine for all pDs would indicate that amino-imino exchange is not the major source to give rise to line broadening of H_8 resonance of this base. On the other hand, we observed the broad-H_8 resonance of 2 N N′ dimethylanimomethylene guanosine. This would imply that the keto-enol exchange might be the only possible one to cause broad H_8 resonance. Because in these two compounds, parts of the tautomeric hydrogens are substituted with

methyl group, only one possible tautomeric exchange can exist in each case.

Having established that keto-enol tautomerism is a source for H_8 line broadening, we may correlate this with the formation of tetramers and proton transfer mechanism Fig.(10). It is reasonable that in the collision and pairing process the formation of tetramer will also catalyze this keto-enol tautomeric exchange. This would explain why there is a minimum linewidth at pD ~ 5.0 for 5′ GMP and guanosine where there is relatively high percentage of tetramers in solution.

If the line broadening of H_8 resonance is from fast chemical exchange at room temperature, its linewidth would be very sensitive to the external magnetic field. Generally speaking the linewidth is related to its resonant frequency and exchange life time by the following approximate expression:

$$\frac{(\Delta\nu)_{100}}{(\Delta\nu)_{220}} = \frac{\left(\frac{1}{T_{2_b}^2} + \frac{1}{\tau_b \tau_{2_b}}\right) + \Delta\dot{\omega}_{100}^2}{\left(\frac{1}{T_{2_b}} + \frac{1}{\tau_b}\right)^2 + \Delta\dot{\omega}_{100}^2} \times \frac{\left(\frac{1}{T_{2_b}} + \frac{1}{\tau_b}\right)^2 + \Delta\dot{\omega}_{220}^2}{\left(\frac{1}{T_{2_b}^2} + \frac{1}{\tau_b \tau_{2_b}}\right) + \Delta\dot{\omega}_{220}^2} \quad (2$$

where $(\Delta\nu)_{100}$ and $(\Delta\nu)_{220}$ are the exchange linewidth measured with HA-100 and HR-220 spectrometers respectively. The exchange linewidth is the actual width originating from chemical exchange and obtained from the observed linewidth by subtracting the natural linewidth. $\Delta\dot{\omega}_{100}$ and $\Delta\dot{\omega}_{220}$ are the chemical shift differences of the two exchange species measured by HA-100 and HR-220 respectively.

τ_b is mean life time of minor species, ie; enol form. T_{2b} is its relaxation time in the absence of chemical exchange. Since the chemical shift is proportional to external magnetic field, the applied magnetic field in HR-220 is 2.2 times higher than in HA-100, ie; $\Delta\dot{\omega}_{220} = 2.2\,\Delta\dot{\omega}_{100}$.

By considering the chemical shift of H_8 resonance of 1 methoxyl derivatives of guanosine, it is possible to estimate the difference in chemical shift between keto and enol tautomers, and the estimated value is about 40 H_z for HA-100 study.

Because the exchange life time τ_b at pD 7.5 for 2'GMP is not very short compared to the inverse of chemical shift difference $(\frac{1}{\Delta\dot{\omega}})$, the exchange line width measured with HR-220 differs from that measured with HA-100 only by a factor of 2.5 but not a perfect square relation (4.8).

From the above evidences and justification, we may conclude that there is tautomeric exchange occurring on the guanine base for some of these compounds and giving rise to line broadening of its H_8 resonance.

At last we also noted that for all the compounds, the coupling constant $J_{H_{1'}H_{2'}}$ varies with pD from 3.8 H_z to 6.2 H_z. This would indicate that the conformation of ribose changes gradually with pD. Since the observed coupling constant is related to the dihedral angle ϕ between $H_{1'}$ and $H_{2'}$ protons by $J_{obs} = J_o \cos^2 \phi - J_1$ where J_{obs} is observed coupling constant, J_o and J_1 are constant. We can calculate the changes in dihedral angle as a function of solution pD.

Kinetic Analysis

Having established qualitatively that the line broadening of H_8 resonances in these compounds is the result of molecular association and tautomeric exchange between bases, it is desirable to extract from our study under different experimental conditions some kinetic and thermodynamic parameters for these processes. We must first derive a proper expression for the H_8 linewidth in each case and from that relation, we can estimate the degree of molecular association, activation energy of tautomeric exchange and the approximate percentage of minor tautomers.

Let's consider a relatively simple case; that the concentration of the solution studied is so low that the effect of molecular association can be neglected, then the H_8 linewidth may predominantly be determined by the effect of monomeric tautomeric exchange.

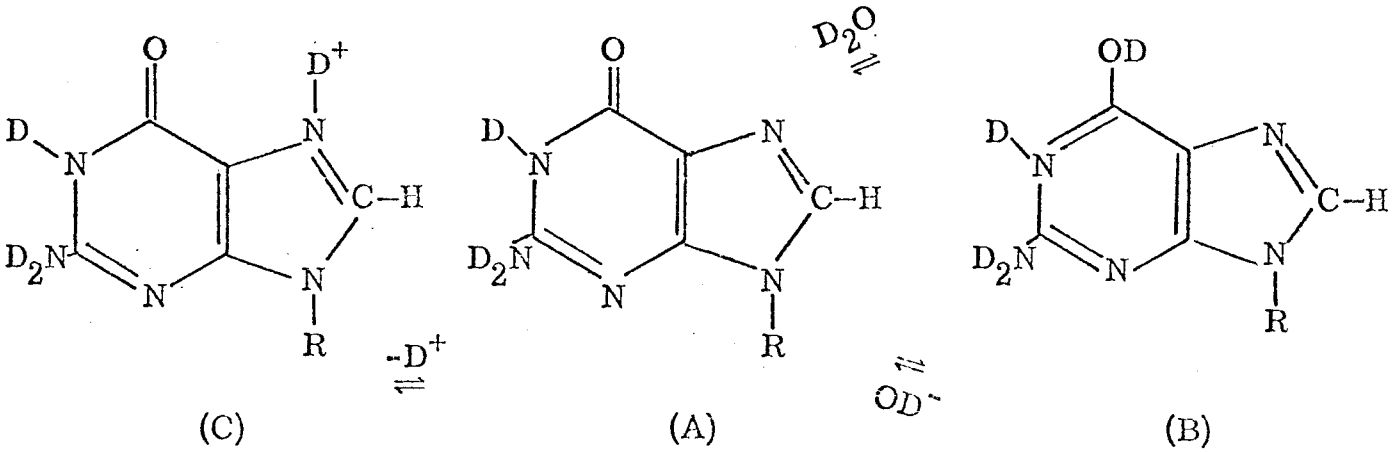
Next we may assume that at low pD, this tautomeric exchange occurs only between two neutral tautomers and the protonation is very fast compared to the tautomeric exchange.

In the study of the tautomeric exchange, we have derived an expression for the observed linewidth based on the three sites exchange treatment. Similarly the same consideration can also be used in the description of tautomeric exchange of guanine base which also involves three exchange species, ie; protonated species and two neutral tautomers.

The observed linewidth of H_8 resonance can therefore be described by Eq. (3).

$$\Delta\nu_{obs} = \left(\frac{1}{\pi}\frac{1}{T_2}\right)_{obs} = \Delta\nu_0 + \frac{P_B^{\,2}}{\pi P_A}\left(1 - P_B\right)^2 \left(\dot{\omega}_{AC} - \dot{\omega}_B\right)^2 \tau_{ab} \qquad (3)$$

where $\Delta\dot{\nu}_0$ is the natural linewidth, $\dot{\omega}_{AC}$ is the average resonance frequency of the protonated and the keto form of base. $\dot{\omega}_B$ is the resonance frequency of enol tautomer. P_A and P_B are the relative populations of two tautomers respectively. τ_{ab} is the average lifetime of keto tautomers exchanged into enol ones. Since the observed linewidth of H_8 resonance is strongly pD dependent, we may assume that the exchange would be catalyzed by solvent (D_2O) or base (OD^-). It seems unreasonable that D^+ is involved in the catalytic reaction, since the protonation occurs prediminately on 7-N positions rather than C-1 keto position.



(C) (A) (B)

where A represents neutral keto tautomer, B represents minor enol tautomer and C is protonated species. The mean lifetime of species A exchanged into B can be expressed in terms of the following kinetic parameters

and concentration of deuterioxyl ion (OD^-).

$$\frac{1}{\tau_{ab}} = \frac{\left(\frac{d[A]}{dt}\right)_f}{[A]} = k_h^a [OD^-] + k_s^a$$

$$= k_1^a e^{-E_h^a/RT} [OD^-] + k_2^a e^{-E_s^a/RT} \qquad (4)$$

where E_s^a and E_h^a represent the activation energy for the solvent and base (OD^-) catalysis respectively, where

$$k_h^a = k_1^a e^{-E_h^a/RT} \quad k_s^a = k_2^a e^{-E_s^a/RT}.$$

Using the same analysis as in the cytosine case, we can also obtain an expression [Eq. (5)] of H_8 linewidth as a function of pD and the other parameters. We may assume that the percentage of enol tautomer is low compared to the keto one, since we couldn't actually observe the H_8 resonance of enol form at low temperature.

$$(\Delta\nu)_{obs} = \Delta\nu_0 + k(\dot{\omega}_{AC} - \dot{\omega}_B)^2 e^{(E_s^a - 2\Delta H)/RT} \frac{[D^+]}{\left\{1 + \frac{[D^+]}{k_a}\right\}\left\{[D^+] + k_3 k_w e^{(E_s^a - E_h^a)/RT}\right.} \qquad (5)$$

where k_a is protonation constant.

$\dot{\omega}_{AC} = (P_A \dot{\omega}_A + P_C \dot{\omega}_C)/(P_A + P_C)$ is the average resonance frequency of protonated species and major keto tautomer. $\dot{\omega}_B$ is the resonance frequency of enol tautomer.

ΔH is the enthalpy difference between two tautomers.

$$k_3 = 10^8 \text{ ExP } [(E_h^a - E_s^a)/0.6] = \frac{k_1^a}{k_2^a} \quad \text{and } k = \frac{1}{\pi} \frac{1}{k_2^a}$$

At extremely low pD, the solvent catalyzes the exchange because the concentration of base (OD^-) is negligible, ie; $k_s^a \gg k_h^a [OD^-]$

$$\frac{1}{\tau_{ab}} \approx k_s^a \approx k_2^a \, e^{-E_s^a/RT} \tag{6}$$

Eq. (5) can be simplified as the following:

$$(\Delta\nu)_{obs} = \Delta\nu_0 + \frac{1}{k_2^a}(\dot{\omega}_{AC} - \dot{\omega}_B)^2 \, e^{(E_s^a - 2\Delta H)/RT} \times \{1 + \frac{[D^+]}{k_a}\}^{-1} \tag{7}$$

By the high temperature study of H_8 linewidth at low pD we may get a linear plot of $\log (\Delta\nu_{obs} - \Delta\nu_0)$ vs $\frac{1}{T}$ with slope $(E_s^a - 2\Delta H)$ as shown in Fig. (8).

The slope calculated for 2′ GMP at pD 3.0 is 9.15 kcal/mole. At high pD ($\geq$ 7.0) the situation is different because there is enough concentration of base (OD^-) with a path of lower activation energy which can catalyze the exchange predominantly, ie; $k_h^a [OD^-] \gg k_s^a$

$$\frac{1}{\tau_{ab}} \approx k_h^a [OD^-] = k_1^a \exp [-E_h^a/RT] [OD^-] \tag{8}$$

Eq. (5) can be simplified as the following:

$$(\Delta\nu)_{obs} = \Delta\nu_0 + \frac{1}{k_1^a k_w} \times (\dot{\omega}_{AC} - \dot{\omega}_B)^2 \, e^{(E_h^a - 2\Delta H)/RT} \, [D^+] \tag{9}$$

From the high temperature study of H_8 resonance, we may also obtain a linear plot of $\log (\Delta\nu_{obs} - \Delta\nu_0)$ vs $\frac{1}{T}$ with slope $E_h^a - 2\Delta H$.

The calculated slope is 3.21 kcal/mole for 2′ GMP at pD 7.2.

At low temperature, if the exchange is in the slow exchange limit, the H_8 linewidth of keto tautomer at high pD may be approximately expressed as in Eq. (10).

$$\Delta\nu_A = \Delta\nu_0 + \frac{1}{\pi}\frac{1}{\tau_{ab}} \approx \Delta\nu_0 + \frac{k_1^a}{\pi} e^{-E_h^a/RT}[OD^-] \tag{10}$$

By measuring the linewidth of H_8 resonance at slow exchange limit we may also get another linear plot of $\log(\Delta\nu_{obs} - \Delta\nu_0)$ vs $\frac{1}{T}$ with slope $-E_h^a$ which may be calculated as 6.31 kcal/mole for 2′GMP at pD 7.2.

According to the above kinetic analysis, we may obtain the following thermodynamic parameters. ΔH = 1.55 kcal/mole, E_h^a = 6.31 kcal/mole, E_s^a = 12.16 kcal/mole.

From ΔH, we can roughly estimate that the percentage of enol tautomer is about 8%.

Another analysis which leads essentially to the same conclusion is now described in the following:

From the field dependence relation of 2′GMP at pD 7.5 we can calculate the life time of enol tautomer (τ_b), ie;

$$\frac{\Delta\nu_{100}}{\Delta\nu_{220}} = \frac{2}{5} = \frac{\left(\frac{1}{T_{2_b}^2} + \frac{1}{\tau_b T_{2_b}}\right) + \Delta\dot{\omega}_{100}^2}{\left(\frac{1}{T_{2_b}} + \frac{1}{\tau_b}\right)^2 + \Delta\dot{\omega}_{100}^2} \times \frac{\left(\frac{1}{T_{2_b}} + \frac{1}{\tau_b}\right)^2 + \Delta\dot{\omega}_{220}^2}{\left(\frac{1}{T_{2_b}^2} + \frac{1}{\tau_b T_{2_b}}\right) + \Delta\dot{\omega}_{220}^2} \tag{11}$$

$$= \frac{\left(\frac{1}{T_2^2} + \frac{1}{\tau_b T_{2_b}}\right) + \Delta\dot{\omega}_{100}^2}{\left(\frac{1}{T_{2_b}} + \frac{1}{\tau_b}\right)^2 + \Delta\dot{\omega}_{100}^2} \times \frac{\left(\frac{1}{T_{2_b}} + \frac{1}{\tau_b}\right)^2 + 4.86\,\Delta\dot{\omega}_{100}^2}{\left(\frac{1}{T_{2_b}^2} + \frac{1}{\tau_b T_{2_b}}\right) + 4.86\,\Delta\dot{\omega}_{100}^2}$$

where $\Delta\dot{\omega}_{220} = 2.2\,\Delta\dot{\omega}_{100}$.

Assuming that $\Delta\dot{\omega}_{100} \approx 40\ H_z \pm 5\ H_z$, we may obtain $\tau_b \approx 2.5 \times 10^{-3}$ sec

Substituting this value into Eq. (1) which is still a good approximation for the fast exchange limit in HA-100 study at room temperature, we may also get the approximate percentage for enol tautomer, ie; 6 ± 2%.

Having established that the kinetic analysis of tautomeric exchange, it is desirable to study how molecular association affects the linewidth H_8 resonance, especially in the intermediate pD region. From the PMR and UV study at various concentrations, we can roughly estimate that the equilibrium constant for molecular associations is about 10^6 for 5′GMP and 10^3 for 3′ and 2′GMP at room temperature. Since we have assumed that the tetramer is the only stable polymeric species which can stack to form helical structure in solution and both the pairing and stacking processes are fast compared to the chemical shift difference between the monomer and polymer states at 30°C. As we have assumed the apparent life time may be expressed as the weight average of the two exchange processes, we may treat this case as an effective two-site exchange problem and substitute τ_{ave} and related quantities into Eq. (3) to obtain a modified linewidth expression, Eq. (12).

$$\begin{array}{ccc} & \text{slow} & \\ G & \rightleftharpoons & G^* \\ \text{fast} \Vert & & \Vert \text{ very fast} \\ G_4 & \rightleftharpoons & G_4^* \end{array}$$

$$K_{eq} = \frac{[G]^4}{[G_4]} = 10^6 \text{ for 5'GMP.}$$

$$P_G + P_{G_4} + P_{G_H} + P_{G^-} + P_{G^*} + P_{G_4^*} = 1$$

Assuming that $P_{G_4^*} \ll (P_{G_4} + P_G + P_{G^-})$,

$$P_{G_4} + P_G + P_{G_H} + P_{G^-} \approx 1$$

$$C_o = 4\,[G_4] + [G] + [G_H^+] + [G^-]$$

where C_o is initial concentration of GMP.

P_G is the population of monomers.

P_{G_4} is the population of tetramers.

P_{G_H} + is the population of protonated monomers.

P_{G^-} is the population of monomer with doubly negative charge on the phosphate group.

P_{G^*} and $P_{G_4^*}$ are their minor enol tautomer.

The modified linewidth expression will be

$$\Delta\nu_{obs} = \Delta\nu_o + \frac{P_{G^*}^2 (1 - P_{G^*})^2}{(1 - P_{G_{H^+}})} (\dot{\omega}_1 - \dot{\omega}_{G^*})^2 (\tau_{av})$$

$$= (1 - \frac{[D^+]}{k_a} P_G)\, e^{-2\Delta H/RT} (\dot{\omega}_1 - \dot{\omega}_{G^*}) (\tau_{av}) \qquad (12)$$

where $\dot{\omega}_1 \approx P_G \delta_G + P_{G_4} \delta_{G_4} + P_{GH^+} \delta_{GH^+} + P_{G^-} \delta_{G^-}$ is the average chemical shift for all keto tautomers.

$$\dot{\omega}_{G^*} \approx \frac{P_{G^{*-}} \delta_{G^{*-}} + P_{G^*} \delta_{G^*}}{P_{G^{*-}} + P_{G^*}}$$ is the average chemical shift for all enol tautomers.

$$\frac{P_G [D^+]}{P_{G_{H^+}}} = k_a$$ where k_a is the deprotonation constant.

$$\frac{P_{G^-} [D^+]}{P_G} = k_b$$ where k_a is the ionization constant of secondary hydrogen on phosphate group.

$$\tau_G^{-1} = k_1 + k_2 [OD^-]$$ where k_1 and k_2 are rate constant.

$$\tau_{ave} = \frac{P_G + P_{G^-} + P_{G^4}}{(P_{G^-} + P_G)\, \tau_G^{-1} + P_{G_4} \tau_{G_4}^{-1}}$$

$$= \frac{C_o - \frac{[D^+][G]}{k_a}}{k_1 [(1 + k_b/[D^+])(1 + 10^{-6}/[D^+]) - (1 + [D^+]/k_a + k_b/[D^+])\, \tau'_{G_4}{}^{-1}]\,[G] + C_o\, \tau'_{G_4}{}^{-1}} \quad (1$$

where we have assumed that the mean life time for proton transfer $\tau_{G_4}^{-1}$ is a function of pD with semi empirical formula given by

$$\tau_{G_4}^{-1} = k_1 \tau'_{G_4}{}^{-1} = 3.0\, k_1 (-pD^2 + 10\, pD - 21.0)$$

The computer simulation using Eq. (12) for the H_8 linewidth is essentially the same as that given by experiment.

There are two reasons with which we can explain the pD dependence of the life time of tautomeric exchange in its tetramer state. At pD 5.0, 5'GMP exists predominately as pure species which is assumed to be the only one forming stable tetramers, while at pD beyond this optimum value, either the protonated species or the species with secondary ionization of phosphate group coexists with the one forming tetramers. The probability of formation of tetramer and the rate of proton transfer will be reduced due to the entropy effect.

As a result, the life time for this proton transfer process in tetramers will become longer at pD higher or lower than 5.0. The entropy effect can also be observed, when we mix 2'GMP with 5' GMP and study at pD ~ 5.0, only one broad H_8 resonance is observed due to the random pairing and slow proton transfer.

From the consideration of equilibrium constant and relatively low percentage of minor enol tautomer, the existence of tetramer in its enol form should be very low compared to its monomers in the ordinary concentration range (10^{-1} - 10^{-2} M). So the overall processes of the chemical exchange in this system would be in a nonequilibrium but steady state.

These two effects would be reasonable to explain the experimental results we have studied in our system.

Conclusion

NMR spectroscopy[5] would be a sensitive and adequate method to study tautomeric exchange of some organic compounds, if their exchange life time and relative populations are within the limit of NMR time scale and sensitivity. From our PMR study of cytosine, guanosine and their related derivatives, we have definitely concluded that there exists at least several percent of minor tautomers for these two biological bases in neutral aqueous solution. As it was suggested by Watson and Crick,[6-8] this would be an important source to induce the gengetic mutation during the DNA replication. Since the percentage of minor tautomer is high relative to the observed spontaneous mutation rate, the kinetic process of replication would be important in controlling this error of incorporation. It would be more interesting if we can study the mutagenic process by using H_5 resonance of cytosine base or H_8 resonance of guanine base as sensitive minitor and investigate its relation to the existence of their minor tautomers.

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FIGURE CAPTIONS

Fig. 1. pD Dependence of Chemical Shifts of H_8 Resonances of Guanosine and Related Compounds.

Fig. 2. pD Dependence of Linewidth of H_8 Resonances of Guanosine and Related Compounds.

Fig. 3. Temperature Contours of Linewidth of H_8 Resonance of 5′ GMP.

Fig. 4. Temperature Contours of Linewidth of H_8 Resonance of 2′ GMP.

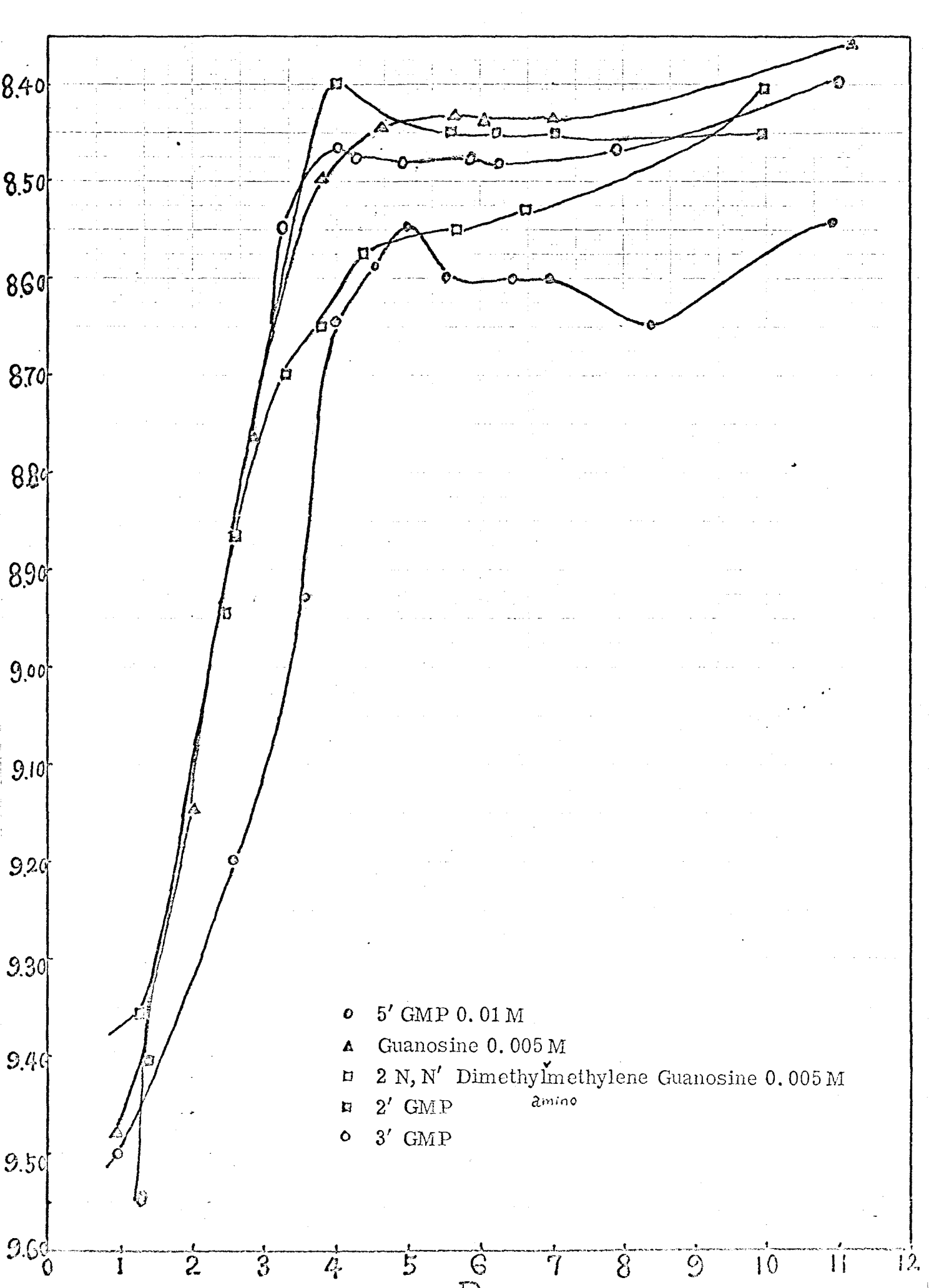
Fig. 5. Concentration Study of Linewidth of H_8 Resonance of GMP Isomers.

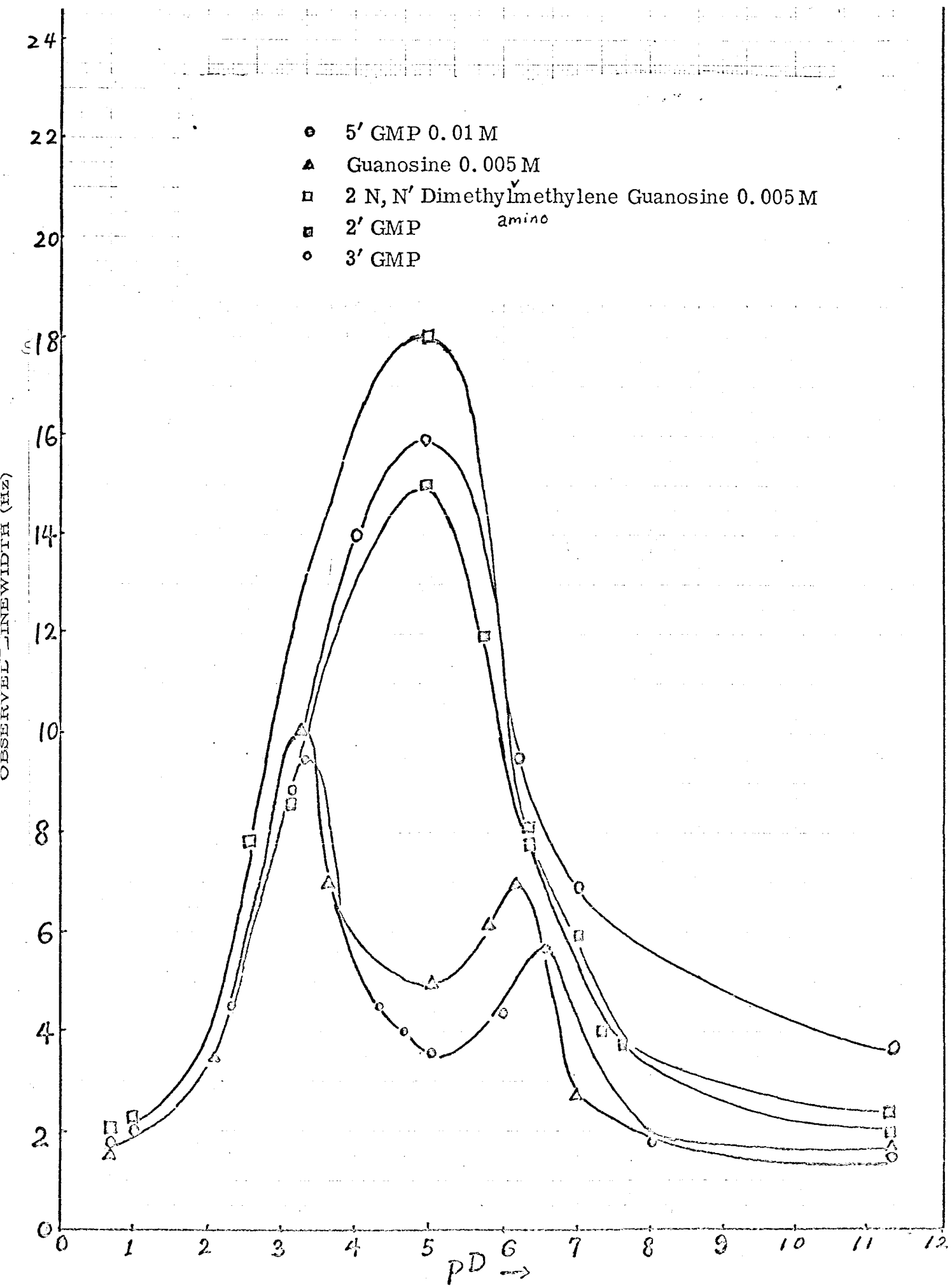
Fig. 6. UV Concentration Study of 5′ GMP.

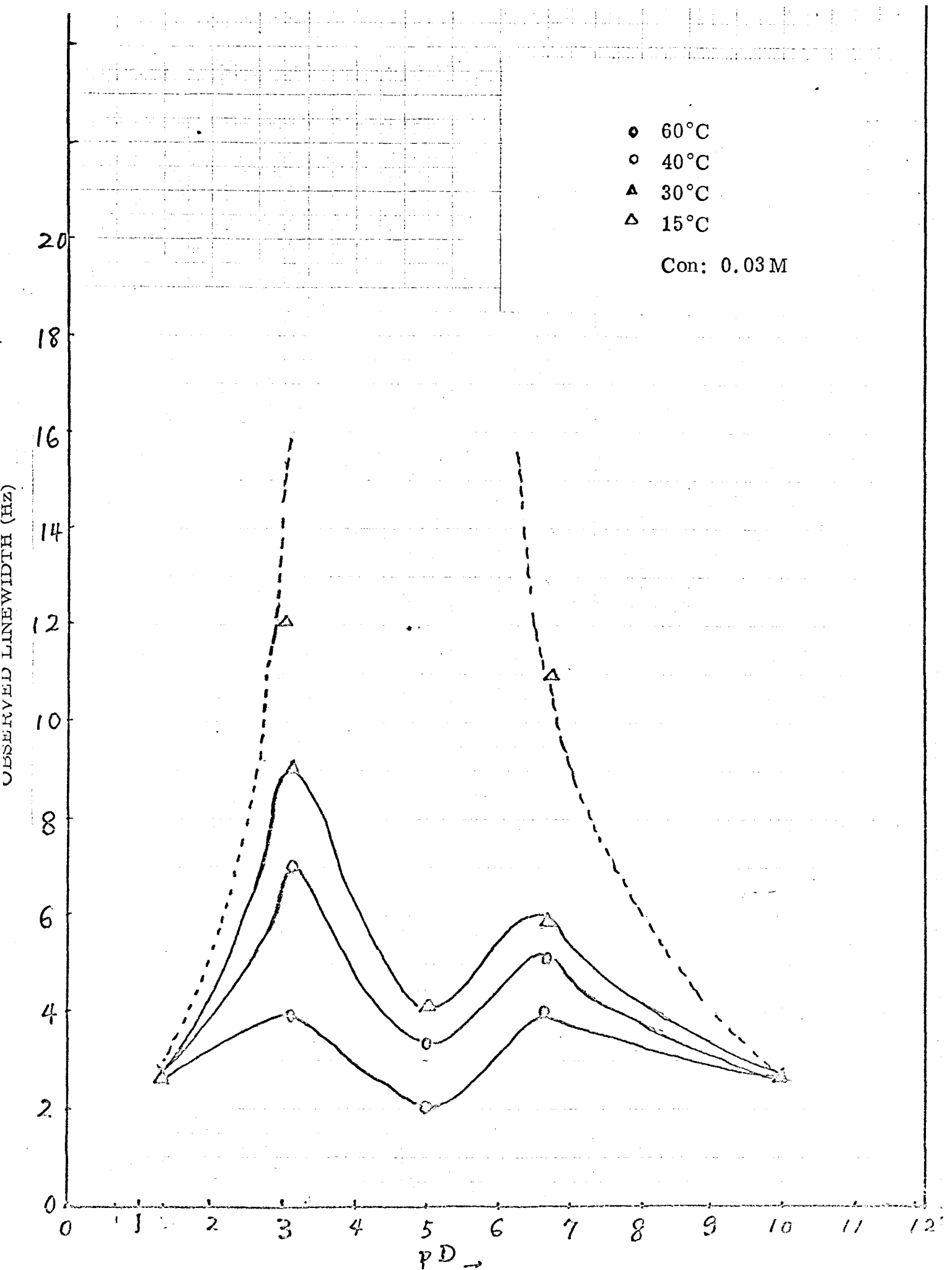
Fig. 7. Viscosity Study of GMP Isomers.

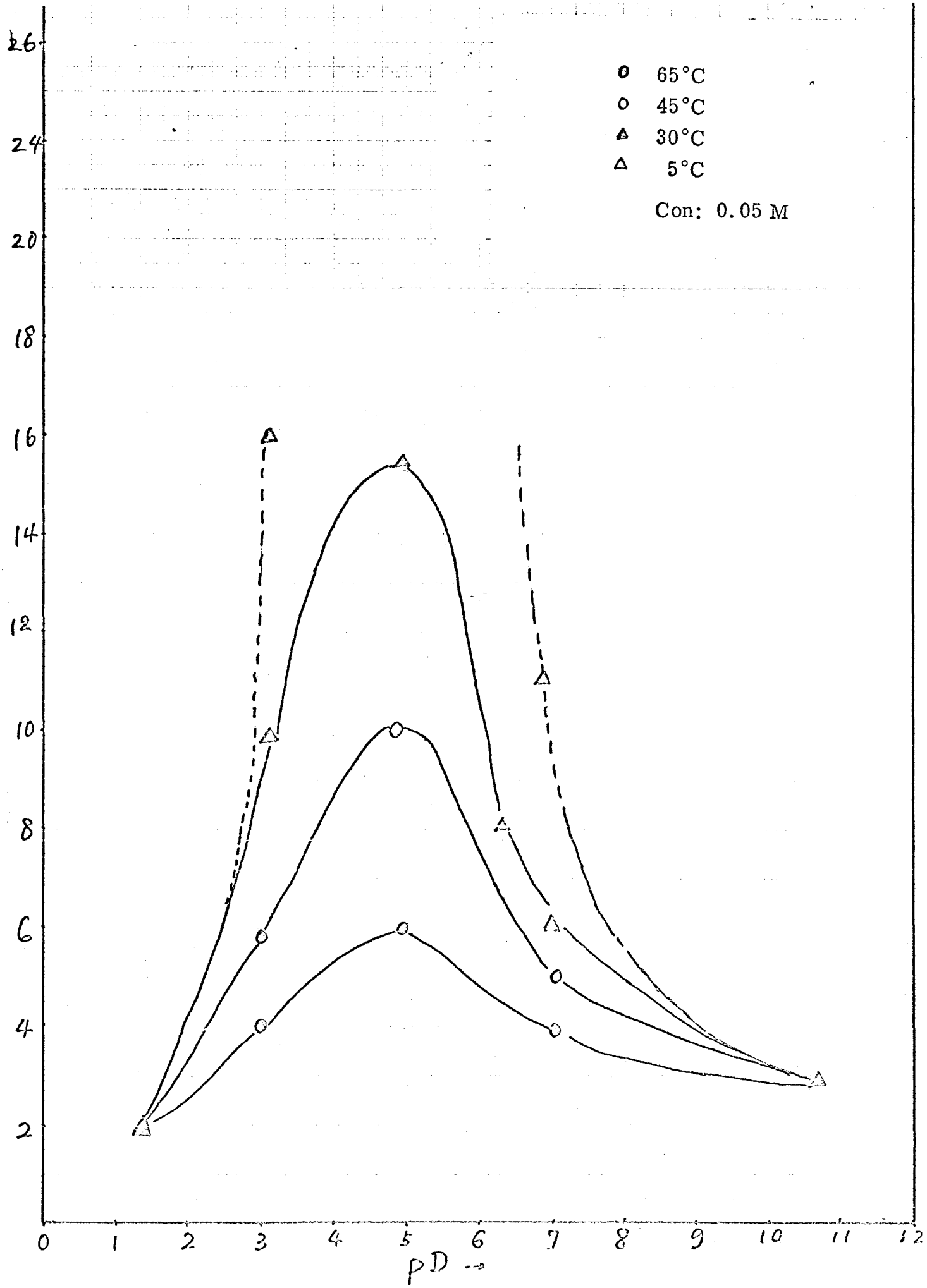
Fig. 8. Temperature Study of Linewidth of H_8 Resonance of 2′ GMP at Different pDs.

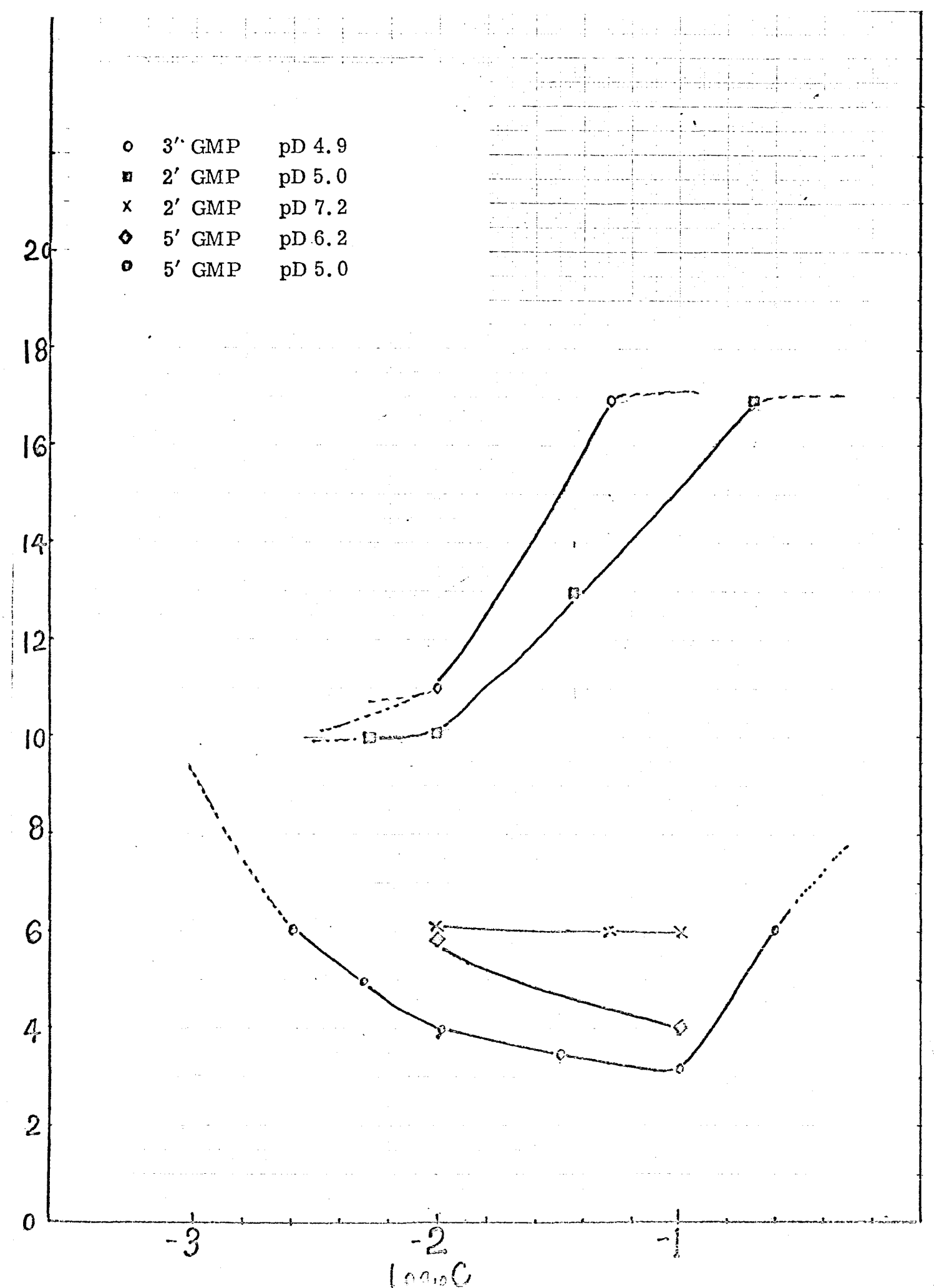
Fig. 9. pD Dependence of Coupling Constant between Ribosal $H_{1'}$ and $H_{2'}$ Protons of Guanosine.

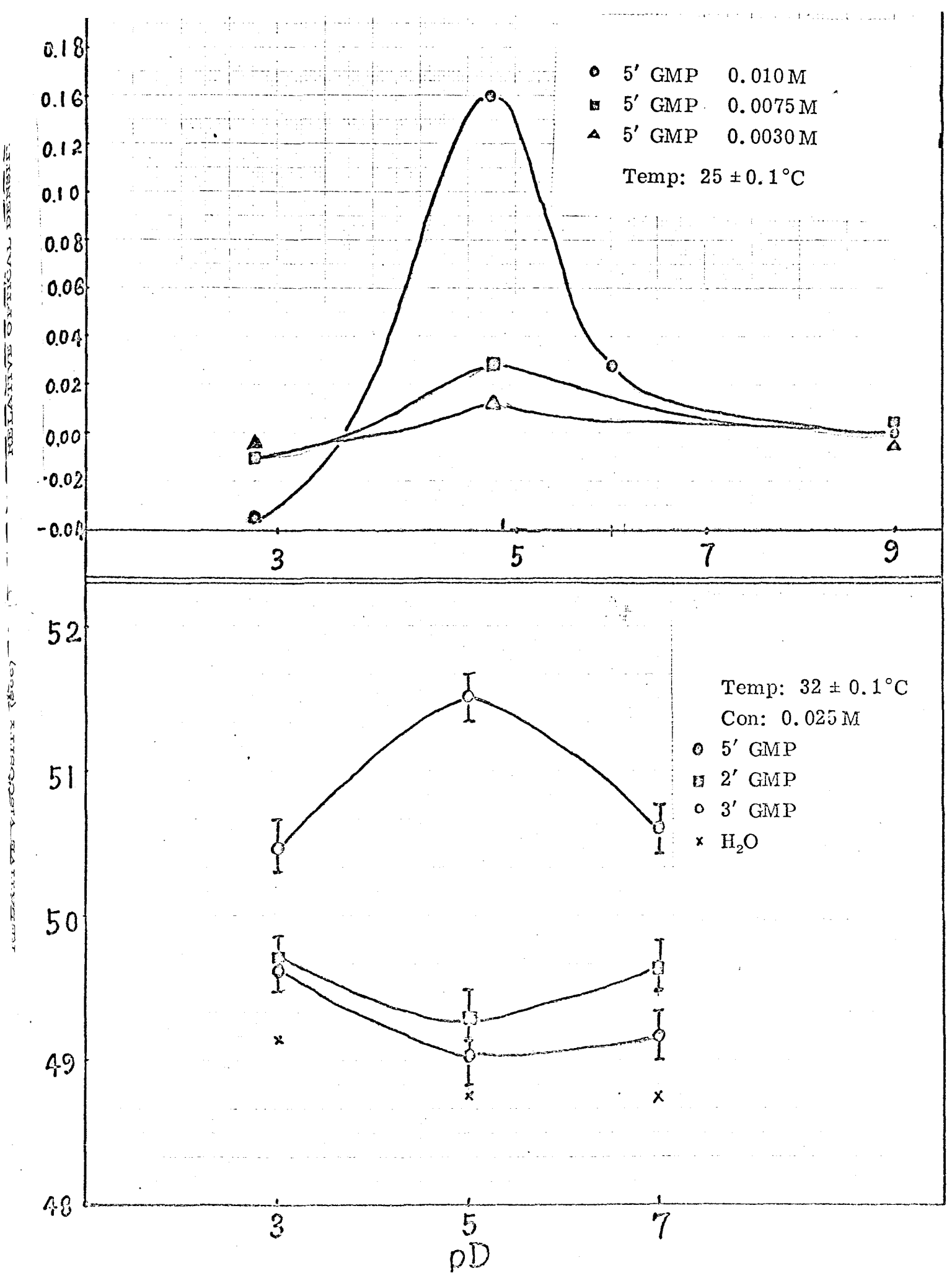

8.40
8.50
8.60
8.70
8.80
8.90
9.00
9.10
9.20
9.30
9.40
9.50
9.60
0
1
2
3
4
5
6
7
8
9
10
11
12
5' GMP 0.01 M
Guanosine 0.005 M
2 N, N' Dimethylmethylene Guanosine 0.005 M
2' GMP
3' GMP

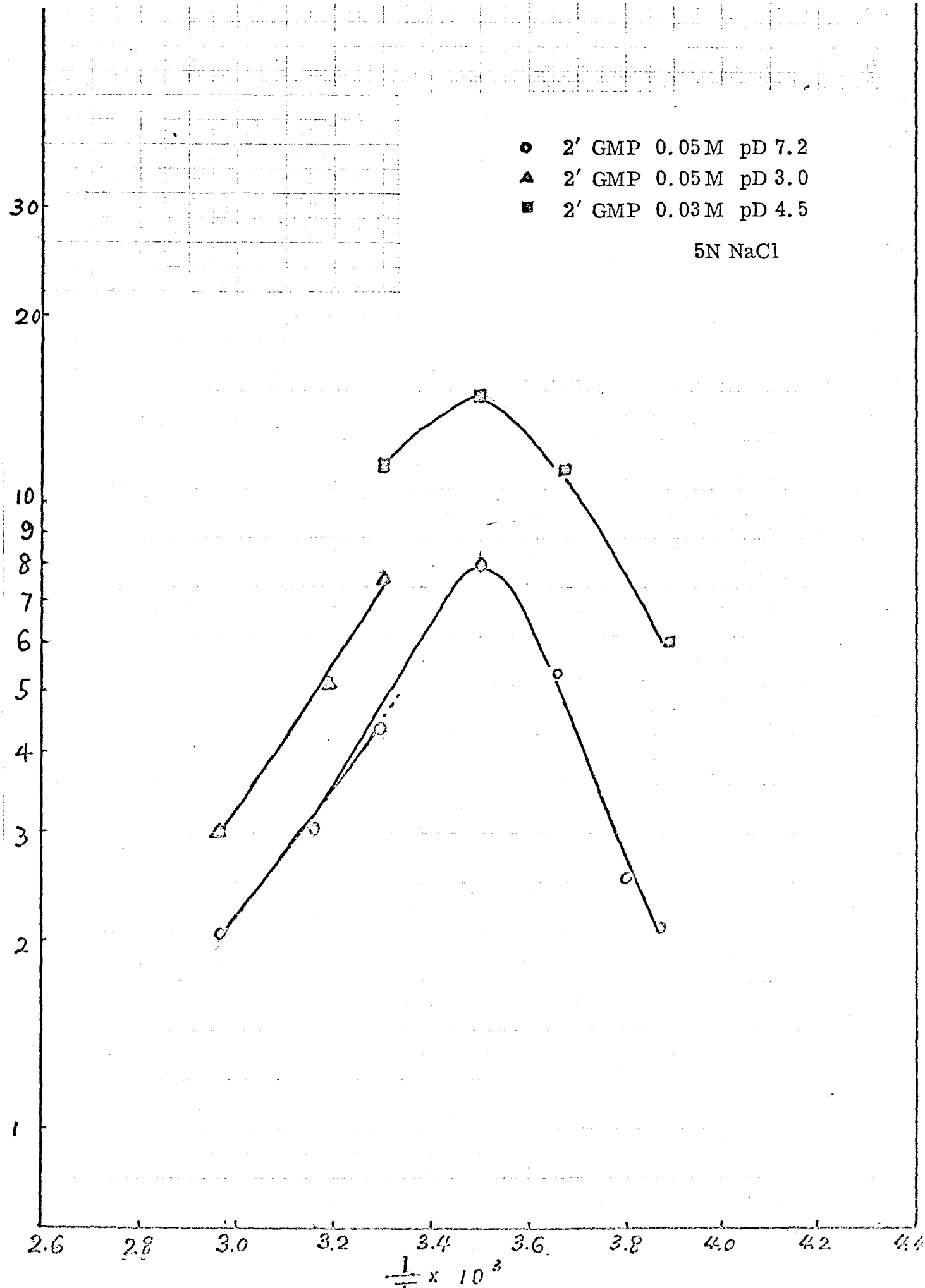

5′ GMP 0.01 M
Guanosine 0.005 M
2 N, N′ Dimethylmethylene amino Guanosine 0.005 M
2′ GMP
3′ GMP
OBSERVED LINEWIDTH (Hz)
pD →
0
2
4
6
8
10
12
14
16
18
20
22
24
0
1
2
3
4
5
6
7
8
9
10
11
12

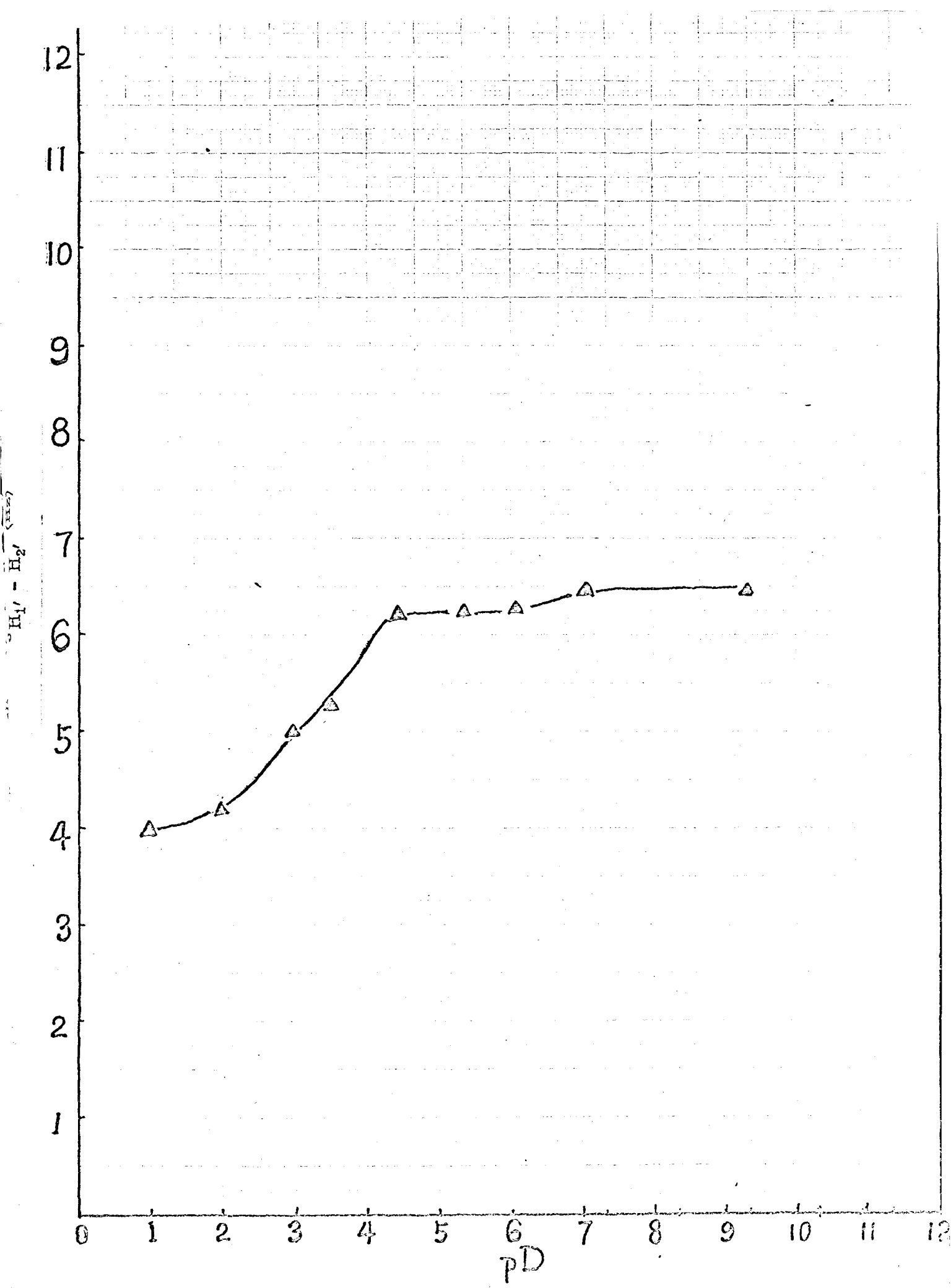

60°C
40°C
30°C
15°C
Con: 0.03 M
OBSERVED LINEWIDTH (Hz)
0
2
4
6
8
10
12
14
16
18
20
0
1
2
3
4
5
6
7
8
9
10
11
12
pD


65°C
45°C
30°C
5°C
Con: 0.05 M
26
24
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
11
12
pD →


o 3′ GMP pD 4.9
2′ GMP pD 5.0
x 2′ GMP pD 7.2
◇ 5′ GMP pD 6.2
5′ GMP pD 5.0
20
18
16
14
12
10
8
6
4
2
0
-3
-2
-1
log10C


0.18
0.16
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
3
5
7
9
5′ GMP 0.010 M
5′ GMP 0.0075 M
5′ GMP 0.0030 M
Temp: 25 ± 0.1 °C
52
51
50
49
48
3
5
7
pD
Temp: 32 ± 0.1 °C
Con: 0.025 M
5′ GMP
2′ GMP
3′ GMP
H2O


2' GMP 0.05M pD 7.2
2' GMP 0.05M pD 3.0
2' GMP 0.03M pD 4.5
5N NaCl
30
20
10
9
8
7
6
5
4
3
2
1
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
$\frac{1}{T} \times 10^3$


12
11
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
11
12
pD