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# Proton Exchange between Nucleic Acid Bases in Nonaqueous Solvents

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**Abstract:** The saturation transfer method of nuclear magnetic resonance reveals that protons exchange between nucleic acid bases in nonaqueous solvents. The exchange takes place between the imino proton of 1-cyclohexyl-5-bromouracil (BrU) and the amino proton of 9-ethyladenine (9EA), and between the amino proton of 1-methylcytosine (1MC) and the imino proton of 9-ethylguanine (9EG), while the amino proton of 9EG does not exchange at all with the other protons. Experiments at low temperature show that, of the two amino protons of cytosine, only the proton directly participating in the hydrogen bond exchanges with the imino proton of 9EG. Activation energies of the proton exchange between some 1-cyclohexyluracil derivatives and 9EA in chloroform were determined. They vary from 7 to 16 kcal/mol, increasing in the following order: 1-cyclohexyl-5-bromouracil, 1-cyclohexyluracil, 1-cyclohexylthymine, and 1-cyclohexyl-5,6-dihydrouracil. It is unlikely that all the protons exchange through water protons in the solvent because the water-proton signal is only partially saturated even when the imino- or amino-proton signals are completely saturated.

The molecular basis of nucleic acid base-base interaction has been studied extensively using various physicochemical techniques including infrared spectroscopy[1] and nuclear magnetic resonance.[2] These experiments demonstrated that a specific interaction between complementary nucleic acid bases exists in nonaqueous solvents even at the monomer level. In spite of much knowledge about specificity, little is known about the dynamic properties of base-pair formation.[3] Specificity or selectivity in base pairing is a fundamental process in genetic coding, but fluctuation in the paired structure should also be relevant to the biological phenomena.[4] In previous work we showed that at the monomer level the adenine–uracil pair using the C(2) carbonyl group as a proton acceptor site coexists extensively with the Watson–Crick type base pair which uses the C(4) carbonyl group.[5] It has also been suggested that the keto-enol tautomerism of thymine (uracil) and guanine and the amino-imino tautomerism of adenine and cytosine may explain spontaneous mutation and wobble pairing in codon-anticodon recognition,[6] although there is almost no experimental evidence supporting the idea. In a previous paper we demonstrated the exchange of protons between complementary nucleic acid bases by applying the saturation transfer method of proton magnetic resonance.[9] The evidence may be relevant to fluctuation in the structure of the bases, particularly tautomerism. Saturation transfer is a well-known phenomenon[7] and has usually been employed to elucidate the sites and rates of exchangeable nuclei.[8] In the present experiment we analyze the observed proton saturation transfer on nucleic acid base pairs in nonaqueous solvents and discuss the mechanism of proton exchange between the bases.

## Experimental Section

**Materials.** 9-Ethyladenine (9EA), 1-cyclohexyluracil (U), 1-cyclohexylthymine (T), 1-cyclohexyl-5-bromouracil (BrU), 1-cyclohexyl-5,6-dihydrouracil (DU), 9-ethylguanine (9EG), and 1-methylcytosine (1MC) were purchased from Cyclo Chemical Co., Los

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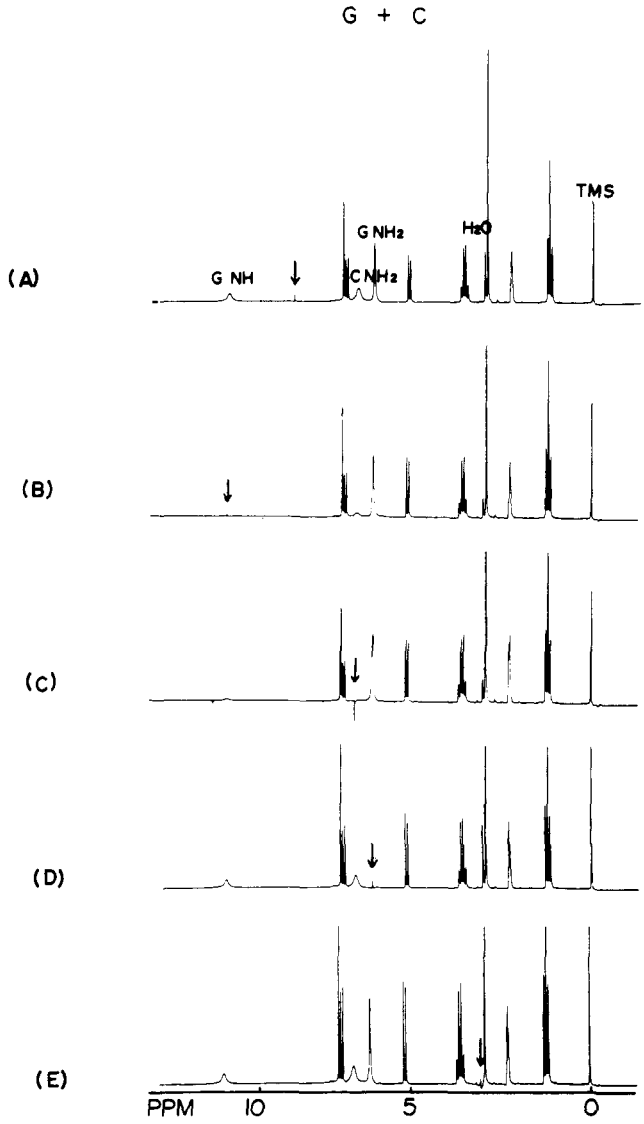


**Figure 1.** $^1H$ spectra of the 1:1 mixture of 9EG and 1MC in dimethyl-$d_6$ sulfoxide at 25 °C. Concentration of 9EG and 1MC is 50 mM. Arrows indicate irradiated positions. The spectra are obtained irradiated (A) at the position where there is no signal, (B) at the imino proton signal of 9EG, (C) at the amino proton signal of 1MC, (D) at the amino proton signal of 9EG, and (E) at the water signal.

Angeles, Calif. 9-Ethyladenine was recrystallized from a combined solvent of carbon tetrachloride and chloroform. All other compounds were used without further purification. The solvents,dimethyl-$d_6$ sulfoxide and chloroform-$d_1$, were obtained from CEA France.

**Methods.** $^1H$ magnetic resonance spectra were obtained at 100 MHz with a JEOL-FX 100 pulse Fourier transform NMR spectrometer locked on deuterium. $^1H(^1H)$ double irradiation experiments were performed using a JEOL homodecoupling unit. Spin-lattice relaxation time $T_1$ was measured using the $(T_d\text{-}\pi\text{-}t\text{-}\pi/2)_n$ pulse sequence, where the condition $T_d \gg 5T_1$ is satisfied and the imino proton of the uracil derivatives is completely saturated. Signal intensity was obtained by integrating the signal area corrected relative to the signal area of tetramethylsilane and using that as the standard. Sample-tube temperature was controlled by a unit equipped with the NMR system. Temperatures were corrected by the chemical shift of the methanol proton and accuracy is within ±1 °C

**Procedures for Exchange Rate Calculation.** We will consider the system where protons are exchanging between two sites A and B of different chemical environments. In the present experiment site A corresponds, for example, to the amino proton of 9EA, and site B to the imino proton of U derivatives. When a strong radio frequency field is applied to the protons at site B, saturating the B magnetization, the following relation holds:[7a]

$$\frac{dM_Z^A(t)}{dt} = \frac{M_A^0 - M_Z^A(t)}{T_{1A}} - \left(\frac{1}{\tau_A}\right)M_Z^A(t) \quad (1)$$

Here $M_Z^A(t)$ is the magnetization at site A at time $t$, $M_A^0$ is the equilibrium magnetization at site A, $\tau_A$ is the lifetime of the protons at site A, and $T_{1A}$ is the intrinsic spin-lattice relaxation time at site A. In the derivation of eq 1, no account was taken of the contribution of the intermolecular dipole-dipole interaction between the protons at site A and those at site B to magnetization.[10] After a pulse sequence $(T_d\text{-}\pi\text{-}t\text{-}\pi/2)_n$ with the B protons completely saturated, magnetization of the A protons at time $t$ is given by[11]

$$M_Z^A(t) = M_Z^A(\infty)\left[1 - 2\exp\left\{-t\frac{\tau_A + T_{1A}}{\tau_A T_{1A}}\right\}\right] \quad (2)$$

This equation is obtained by solving eq 1 with the boundary condition $M_Z^A(0) = -M_Z^A(\infty)$ and applying the relation[7a]

$$\frac{M_Z^A(\infty)}{M_A^0} = \frac{\tau_A}{\tau_A + T_{1A}} \quad (3)$$

where $M_Z^A(\infty)$ is the magnetization at site A at infinite time after applying the strong radio frequency field at site B and $M_A^0$ is obtained as the magnetization of site A without irradiation at site B. In eq 2 $\tau_A T_{1A}/(\tau_A + T_{1A}) = T_{app}$ corresponds to $T_1$ in the measurement of spin-lattice relaxation times by the normal inversion recovery method.[12] From eq 2 and 3, $\tau_A$ and $T_{1A}$ can be calculated. The exchange reaction in our experiment is expressed as

$$\text{A-H}^* + \text{U-H}^{**} \underset{}{\overset{k}{\rightleftarrows}} \text{A-H}^{**} + \text{U-H}^*$$

where A and U denote adenine and uracil derivatives, and $k$ is the rate constant for the forward or backward exchange process. The lifetime $\tau_A$ is related to $k$ as follows:

$$\tau_A = 1/k[\text{U}] \quad (4)$$

where [U] is the concentration of the uracil derivative. Using the above relation, the rate constant $k$ can be estimated from the concentration dependence of the lifetime $\tau_A$.

## Results

**Guanine–Cytosine Mixture.** Proton magnetic resonance spectra of the 1:1 mixture of 1MC and 9EG in dimethyl-$d_6$ sulfoxide are shown in Figure 1. The signals of the imino proton of 9EG and those of both the amino protons of 1MC and 9EG shift considerably downfield on mixing.[2a] The shifts can be attributed to the formation of hydrogen bonds between 1MC and 9EG. Irradiation on the imino proton of 9EG induces a decrease in intensity of the amino-proton signal of 1MC. Similarly, irradiation on the amino proton of 1MC weakens the imino-proton signal of 9EG. The nuclear Overhauser effect due to dipolar coupling should produce signal enhancement in cases like the present where gyromagnetic ratios of the interacted nuclei are of the same sign[13a] and the molecular motion of the system is rapid.[13b] Thus we can explain the observed intensity decrease as follows. Protons exchange back and forth between the amino group of 1MC and the imino group of 9EG, and the irradiated protons, i.e., the completely saturated protons, move to the nonirradiated site (saturation transfer). As a result, magnetization at the nonirradiated site decreases, if magnetization recovery at the nonirradiated site is slow enough. Irradiation on the amino proton of 9EG had almost no effect on the peak intensities of the other protons. Similarly the signal of the amino proton of 9EG was not affected by irradiation on the other protons. These results indicate that the amino proton of 9EG does not exchange with the other protons.

Although dried dimethyl sulfoxide was used in the experiment, the solvent still contained a slight amount of water. In fact a small water resonance appears in the spectrum from which we can estimate the amount of water contained (Table I). Thus it is necessary to take into account exchange via water molecules. Irradiation on the water resonance weakens the intensity of the imino-proton signal of 9EG to a certain extent, but the percentage decrease is very small compared with the

**Table I.** Percent Decrease in Signal Intensity on Irradiation of Other Proton Signals at 25 °C

| base pairs | solvent (concn) | irradiated proton | % decrease in signal intensity $[M_A^0 - M_Z^A(\infty)]/M_A^0(\infty) \times 100$ G-amino | C-amino | A-amino (2) | A-amino (6) | G-imino | U-imino |
|---|---|---|---|---|---|---|---|---|
| 9-ethylguanine | $Me_2SO$ | G-amino | | 12 | | | 20 | |
| + | (0.1 M | C-amino | 12 | | | | 86 | |
| 1-methylcytosine | (0.042 M)[a] | G-imino | 0 | 71 | | | | |
| | | $H_2O$ | 3 | 5 | | | 20 | |
| 9-ethyladenine | $CDCl_3$ | A-amino | | | | | | 71 |
| + | (0.2 M)[b] | U-imino | | | | 41 | | |
| 1-cyclohexyl-5-bromouracil | | $H_2O$ | | | | 33 | | 58 |
| | $Me_2SO$ | A-amino | | | | | | 65 |
| | (0.2 M) | U-imino | | | | 32 | | |
| | (0.052 M)[a] | $H_2O$ | | | | 17 | | 61 |
| 2,6-diamino-9-ethylpurine | $Me_2SO$ | A-amino(2) | | | | 8 | | |
| | (0.2 M) | A-amino(6) | | | 0 | | | |
| | (0.033 M)[a] | $H_2O$ | | | 2 | 1 | | |
| 2,6-diamino-9-ethylpurine | $Me_2SO$ | A-amino(2) | | | | 12 | | 43 |
| | (0.2 M) | A-amino(6) | | | 12 | | | 58 |
| + | (0.060 M)[a] | U-imino | | | 17 | 24 | | |
| 1-cyclohexyl-5-bromouracil | | $H_2O$ | | | 21 | 19 | | 75 |

[a] Molar concentration of water estimated from signal intensity. [b] Water signal is too broad to calculate the amount of water.

case of irradiation on the amino proton of 1MC (Table I). Protons of the water molecules certainly exchange with the amino protons of 1MC and the imino proton of 9EG, but direct exchange between the imino protons of 9EG and the amino protons of 1MC is predominant in this system.

In the next step, we tried to find which proton of the amino group of 1MC exchanges more easily. It has been known that the amino group of 1MC forms a large potential barrier for rotation about the C(4)–N(amino) bond and the two protons of the group are observed as separate signals at low temperature.[2a] One at the lower field corresponds to the signal of the proton directly participating in the hydrogen bond with 9EG and the other in the upper field corresponds to that of the proton free from the hydrogen bond (Figure 2). The assignments were confirmed by observing the concentration dependence of the two peaks.[14] Under these conditions, the irradiation on the imino proton of 9EG produced saturation more effectively on the proton signal in the lower field, i.e., the signal of the amino proton directly participating in the hydrogen bond (Figure 2). Irradiation on the lower field amino proton also decreased more effectively the intensity of the imino-proton signal of 9EG (44%) than irradiation on the upper field amino proton (29%). These observations indicate that the amino proton participating in the hydrogen bond exchanges more easily with the imino proton of 9EG. The amino proton free from the hydrogen bond can also exchange with the imino proton to a slight extent. It is very probable that the saturated free amino proton moves to the hydrogen-bonded site through rotation about the C(4)–N(amino) bond and exchanges with the imino proton of 9EG. When the free amino proton is irradiated, the bonded amino proton signal decreases by 40% and the imino proton signal of 9EG does so by 29%. About 18% of the 29% decrease is estimated to be contributed by saturation transfer to the imino protons through the passing of the bonded amino proton, because the efficiency of direct saturation transfer between the bonded amino and imino protons is 44% as mentioned above. The residual intensity decrease (11%) may come from the exchange via water and direct exchange between the free amino proton and the imino proton.

**Adenine–Uracil Mixtures.** Similar experiments were performed in a mixture of 9-ethyladenine and 1-cyclohexyl-5-bromouracil. In Table I the intensity decreases are shown for cases of irradiation on other exchangeable protons. The effects of irradiation on the amino-proton signal of 9EA induced a

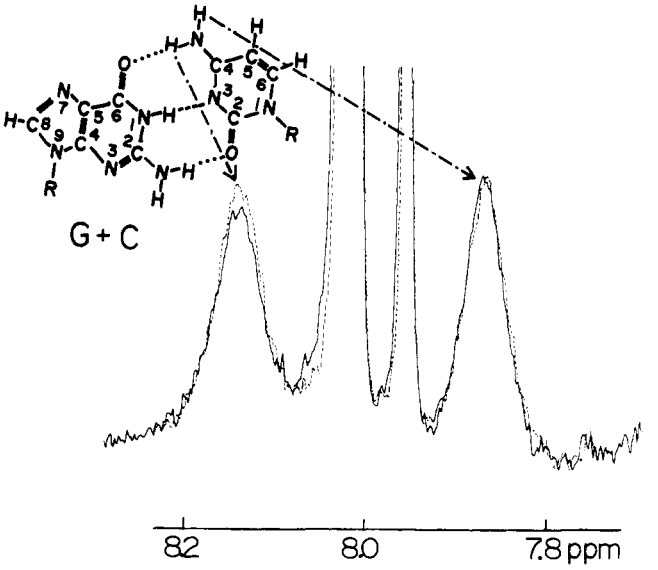


**Figure 2.** $^1H$ spectra of the 1:1 mixture of 9EG and 1MC in a mixed solvent of dimethyl-$d_6$ sulfoxide and chloroform-$d_1$ (1:1) at 3 °C (0.05 M). The solid line shows the normal spectrum and the dotted line is the spectrum where the imino-proton signal of 9EG is irradiated.

decrease in the imino-proton signal of BrU in chloroform solution. However, irradiation on the broad signal of water at 3 ppm, which was overlooked in the previous paper,[9] also induces a notable decrease of intensity in both imino- and amino-proton signals. Thus there seems a considerable degree of proton exchange between the imino proton and the amino proton via water molecules. In dimethyl sulfoxide solution the proton exchange decreases. In this system, however, exchange via water molecules is reduced and direct exchange between imino proton and amino proton becomes predominant, because irradiation on the water signal does not influence imino- and amino-proton signals.

In the spectrum of the mixture solution of 2,6-diaminopurine (DiA) and BrU, separate signals corresponding to the two amino groups are observed. Irradiation on the imino proton of BrU and $H_2O$ shows about the same degree of saturation of the protons at the two amino groups in DiA. However, in the DiA solution itself, irradiation on the $H_2O$ signal does not

**Table II.** Proton Exchange Rate Constants between 9-Ethyladenine and 1-Cyclohexyluracil in Chloroform-$d_1$ (0.2 M Solution)

| temp, °C | intensity ratio $M_Z^A(\infty)/M_A^0$ | $T_{app}$ | $T_{1A}$, s | $1/\tau_A$, $s^{-1}$ | $k$, $s^{-1}$ $M^{-1}$ |
|---|---|---|---|---|---|
| −5 | 0.86 | 0.12 | 0.14 | 2.4 | 12 |
| 4 | 0.79 | 0.12 | 0.15 | 3.6 | 18 |
| 15 | 0.61 | 0.12 | 0.20 | 6.5 | 33 |
| 26 | 0.44 | 0.10 | 0.23 | 11 | 55 |
| 43.5 | 0.26 | 0.06 | 0.23 | 25 | 125 |

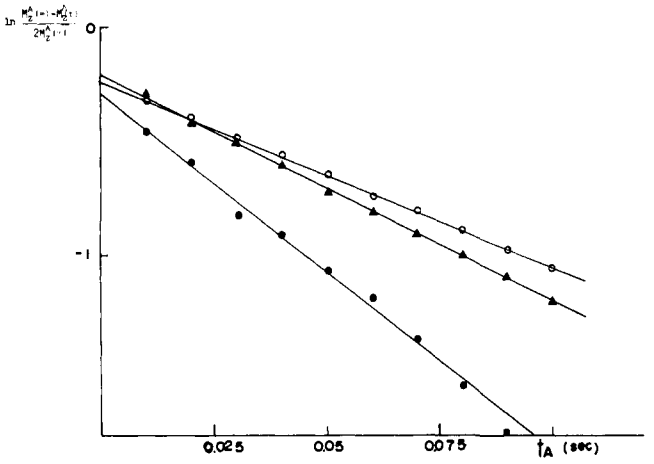


**Figure 3.** A plot of ln $[(M_Z^A(\infty) - M_Z^A(t))/2M_Z^A(\infty)]$ against $t$ for BrU and 9EG system: ○, at −5 °C; ▲, at 26 °C; ●, at 43.5 °C.

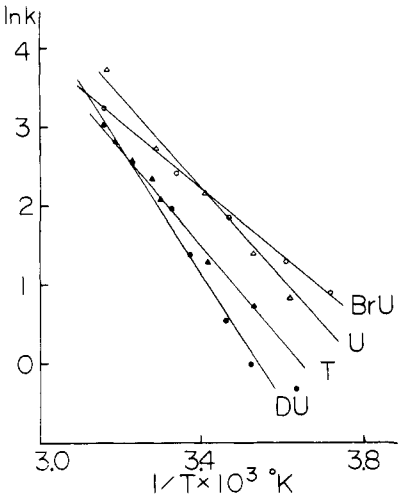


**Figure 4.** Arrhenius plots of the proton exchange rate constants of BrU-9EA (○), U-9EA (△), T-9EA (▲), and DU-9EA (●) systems.

weaken amino proton signals of DiA. Therefore the amino protons of DiA do not directly exchange with water protons and the decreased amino-proton signal in the mixture solution is attributed to the exchange via the imino protons of BrU ($H_2O \rightarrow$ imino proton of BrU $\rightarrow$ amino proton of DiA). Direct exchange between the imino proton of BrU and the amino protons of DiA should exist in this system. An interesting aspect of this system is that the lower field signal of the two amino groups, which is assigned to the 6-amino group, is much more affected than the upper field signal of the 2-amino group by irradiation on the imino proton of BrU.

The rate constants and the activation energies of the proton exchange in the system of 9EA and U derivatives (BrU, U, T, and DU) were obtained by the above method. Under the saturation of the imino-proton signals of the U derivatives in the mixture with 9EA, the intensity of the amino-proton signal of 9EA was measured at ten different intervals ($t$) in the ($T_d$-$\pi$-$t$-$\pi/2$) pulse sequence. If the assumption in the derivation of eq 2 is valid, the plot of ln $(M_Z^A(\infty) - M_Z^A(t))/2M_Z^A(\infty)$ against $t$ should be linear. Such is the case in the 9EA-BrU system as shown in Figure 3, where plots of 43.5, 26, and −5 °C are given. The rate constants of proton exchange were obtained from the slopes of the graphs and the ratios of $M_Z^A(\infty)/M_A^0$ (Table II). It indicates that proton exchange occurs about ten times a second at room temperature. From the temperature dependence of the proton-exchange rate constants, the activation energies of the proton exchange between 9EA and U derivatives were calculated (Figure 4, Table III). Activation energy increases in the order BrU, U, T, and DU.

## Discussion

We presented here evidence of proton exchange between nucleic acid bases in nonaqueous solvents obtained from experiments of saturation transfer in NMR. It has become clear that two conditions are necessary for effective proton exchange between the nucleic acid bases. One is the formation of hydrogen-bonded base pairs and the other is that the exchanging protons are located in the cyclic hydrogen bond system where keto-enol tautomerism can take place.

**Table III.** Activation Energies of Proton Exchange in Mixture Systems of 1-Cyclohexyluracil Derivatives and 9-Ethyladenine

| compd | $\Delta E$, kcal/mol |
|---|---|
| dihydrouracil | 15.3 ± 1 |
| thymine | 13.8 ± 0.2 |
| uracil | 12.5 ± 0.05 |
| 5-bromouracil | 7.8 ± 0.2 |

When we compare the extent of saturation transfer between the dimethyl sulfoxide and chloroform solutions of the 9EA and BrU mixtures, proton exchange occurs more effectively in the chloroform solution than in the $Me_2SO$ solution. Generally, hydrogen bonding between the solute molecules in dimethyl sulfoxide solution is less than in chloroform solution. The association constant between 9EA and BrU in chloroform is bigger than that in dimethyl sulfoxide. As shown in Table III, the BrU mixture has the lowest activation energy for proton exchange and the DU mixture the highest. The association constant between 9EA and BrU is the biggest and that between 9EA and DU the smallest among those of the mixtures of the uracil derivatives with 9EA.[1d] Thus readiness of proton exchange is correlated with the association constants. But we cannot deny that the water molecules also have an affinity to the uracil derivatives in parallel with the association constants with 9EA. More direct evidence to support the hypothesis that the formation of hydrogen bonds is necessary for proton exchange is the fact that among the two amino protons of 1MC the proton participating in the hydrogen bond exchanges more effectively with the imino proton of 9EG.

In the mixture system of 9EG + 1MC, the 2-amino proton signal in 9EG was hardly affected by irradiation of imino protons. We can write the keto-enol (amino-imino) tautomeric

structure of 9EG + 1MC, 9EA + BrU, and DiA + BrU using the amino groups of 1MC, 9EA, and DiA, while the tautomeric form using the 2-amino group of 9EG cannot be written down. The order of activation energies in proton exchanges of uracil derivatives may be relevant to their ease in assuming the enol form. A previous study on tautomerism revealed that 5-bromouracil assumes an enol structure more easily than thymine.[15]

Based on the above data we present possible mechanisms for proton exchange between the complementary base pairs. They are conjectures and do not follow rigorously from the experiments. Firstly, when 9EG forms a hydrogen-bonded dimer with 1MC, the amino proton of 1MC moves to the carbonyl group of 9EG and the imino proton of 9EG to the 3N position of 1MC. 1MC and 9EG then become imino and enol forms, respectively. The possibility of such double tunneling in the base pair was pursued theoretically by Lowdin[16] and the existence of the imino form of cytosine derivatives has been demonstrated.[17] To interpret the proton exchange between the amino group of 1MC and the imino group of 9EG, the recovery to the normal tautomeric form through rearrangement of the moved protons should occur inside the molecule (Figure 5E). It is not clear whether such an inner-proton jump is possible. In this mechanism it does not matter whether the hydrogen-bonded pair dissociates after the double tunneling. However, the lifetime of the base pair in chloroform is said to be of the order of $10^{-8}$ s.[3] The contribution of the dissociated species to the proton exchange should then be considered. For example, after the double tunneling, they dissociate, keeping the imino-enol tautomeric forms, and then they may form self-associated dimers which would easily induce recovery to the normal tautomeric forms (Figure 5D).

Another probable mechanism is the formation of protonated species instead of double tunneling. Formation of a hydrogen-bonded pair may allow proton transfer of the imino proton of 9EG to the counterbase, 1MC.[18] After dissociation of the base pair the amino group of the protonated 1MC may directly attack the deprotonated imino group of 9EG in random collisions and the rearrangement of exchangeable protons may occur inside the 1MC. This would also explain the proton exchange between the imino group of 9EG and the amino group of 1MC.

At the present stage we cannot determine the actual mechanism of the observed proton exchange, but at least it is clear that such exchanges occur without water molecules and also that the formation of the base pair is necessary for the exchange. If the contribution of the dissociated species from the base pair is essential for proton exchange, such an exchange occurs scarcely at all or is at a low rate in the double-stranded nucleic acids. An experiment on the polynucleotides would help the interpretation and the results would also make clear whether the phenomenon has some relevance to mutations.

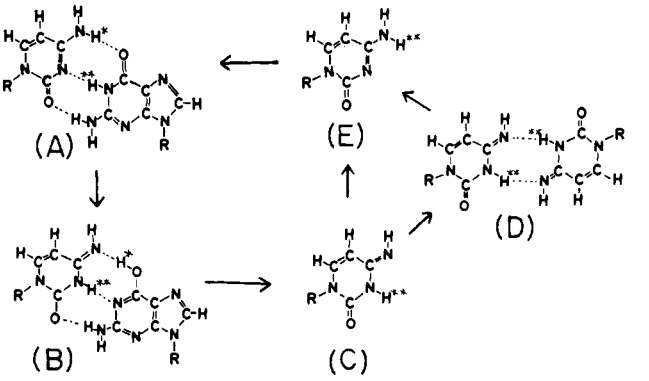


**Figure 5.** One possible mechanism for proton exchange.

## References and Notes

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