Mechanism of the Isotopic Exchange of the C-8 Hydrogen of Purines in Nucleosides and in Deoxyribonucleic Acid†

Maria Tomasz,* Janet Olson, and Carmen M. Mercado

ABSTRACT: The kinetics of the exchange of tritium in the C-8 position of guanosine, 1-methylguanosine, adenosine, and guanine residues of DNA were determined at various pH values at 37°. At constant pH pseudo-first-order kinetics were observed in all cases. The rate of the exchange reaction is completely independent of pH in the wide pH ranges employed with the exception of guanosine which shows a rate increase in the pH range of about 7–11, similar in shape to the titration curve of the N-1-H group of guanosine. Catalysis by buffer anions was not observed in the compounds tested (guanosine and adenosine). The pH-independent kinetics are best interpreted by postulating that the mechanism of the exchange reaction consists of the OH^--catalyzed abstraction of the C-8 hydrogen ion from the 7-protonated form of the purines giving rise to an ylid type intermediate which is then reprotonated at C-8 by the medium. This type of mechanism has been typically observed in imidazoles and other related 5-membered heterocyclic systems by other investigators. The "anomalous" rate boost by alkali in the case of guanosine is explained by the following scheme: in addition to the main pH-independent mechanism a second pathway of exchange exists involving the participation of a new tautomeric form of guanosine: the guanosine zwitterion. Its tautomeric equilibrium constant, calculated from pK_a data, is 1.9×10^{-5} at 37° and at 0.1 ionic strength. It is proposed that this form may be considered as a "rare tautomer" capable of mispairing in the course of DNA replication.

The phenomenon of isotopic proton exchange at the C-8 position of purines of nucleic acids, nucleosides, and nucleotides has received considerable attention in recent years. The initial observations in this area were made by Ts'o and his collaborators (Schweizer *et al.*, 1964) and by Bullock and Jardetzky (1964). Using nuclear magnetic resonance (nmr) techniques both groups noted the facile exchange of the C-8 proton of purines in D_2O at elevated temperature. Other workers observed analogous behavior of purine residues in DNA (Ostermann *et al.*, 1966; Fritzsche, 1967). Shelton and Clark (1967) reported a more extensive investigation of the exchange reaction employing nucleosides in $[^3H]H_2O$ as their model system. As an extension of the model studies to practical application the same authors (1968) and also others (Searcy, 1968; Doppler-Bernardi and Felsenfeld, 1969) reported the labeling of DNA with tritium *in vitro* simply by heating it in $[^3H]H_2O$ to allow incorporation of tritium in the C-8 position of the purine residues. DNA labeled in this manner is suitable for hybridization studies and thus the method has great potential use, especially for nucleic acids of higher organisms since methods for labeling them *in vivo* are not usually feasible.

While the mentioned investigations revealed a great deal of important information on the nature of the exchange reaction its actual mechanism remained unexplored. The purpose of the present work was to elucidate this problem.

Experimental Section

Materials. [8-^{3}H]Guanosine (5.3 Ci/mmole; "greater than 99% of ^{3}H is located at position 8") was obtained from New England Nuclear Corp., Boston, Mass., and [8-^{3}H]adenosine (28 Ci/mmole) from Schwarz BioResearch, Orangeburg, N. Y. Both of these substances were used undiluted by cold nucleosides.

[8-^{3}H]-1-Methylguanosine was synthesized by methylation of [8-^{3}H]guanosine with CH_3I according to the procedure of Broom *et al.* (1964) described for nonradioactive synthesis. The starting material [8-^{3}H]guanosine was thoroughly diluted with cold guanosine; the specific activity of the methylated product was 0.63 mCi/mmole.

^{3}H-Labeled bacterial DNA continuing 83% of the total radioactivity in the 8 position of guanine and 17% in the adenine residues was prepared by biosynthetic labeling with [8-^{3}H]guanosine as described in a previous paper (Tomasz, 1970a); its specific radioactivity was 6.38×10^5 dpm per A_{260} unit. (Absorbance of DNA was measured in 0.15 M NaCl–0.015 M Na citrate buffer, pH 7.8.) Denatured DNA was prepared by treatment with 95% formamide (Marmur and Ts'o, 1961).

Methods. DETERMINATION OF THE PSEUDO-FIRST-ORDER RATE CONSTANTS (k_ψ'S) OF THE ^{3}H-EXCHANGE REACTIONS. Aliquots of [8-^{3}H]purine nucleosides (50,000–200,000 cpm) or [8-^{3}H]purine-containing DNA (approximately 20,000 cpm, 0.3 A_{260} unit) were incubated in 1.0-ml buffers of appropriate pH containing a drop of $CHCl_3$ at 37.0° for various time periods (6–24 hr; longer in the case of DNA.) The extent of release of tritium into the medium was assayed by distilling the sample to dryness *in vacuo* followed by counting the radioactivity of the distillate, *i.e.*, $[^3H]H_2O$, in Bray's solution as described in detail in a previous paper (Tomasz, 1970b). Since stock solutions of the substrates undergo slow ^{3}H release on standing even in the refrigerator a "0-time" assay was included for correction for each series. Total initial ^{3}H content of the substrates was determined by heating them in a

† From the Department of Chemistry, Hunter College, The City University of New York, New York, New York 10021. *Received October 11, 1971.* This work was supported by the U. S. Public Health Service (AI-08325) and the Health Research Council of the City of New York (U-2039), and a Faculty Research Award from the City University of New York.

[1] At $K_{a_1} \gg [H^+] \gg K_{a_2}$ (approximate pH 4–6) $k_\psi^{(1)} \cong k_1 K_w / K_{a_1}$, *i.e.*, constant while $k_\psi^{(2)} \cong (k_2 K_{a_1})(K_w / K_{a_1}[H^+])$, *i.e.*, increasing with increasing pH. The fact that according to the experimental data k_ψ (the sum of $k_\psi^{(1)}$ and $k_\psi^{(2)}$) is *constant* in this region indicates that the increasing term $k_\psi^{(2)}$ must be negligibly small.

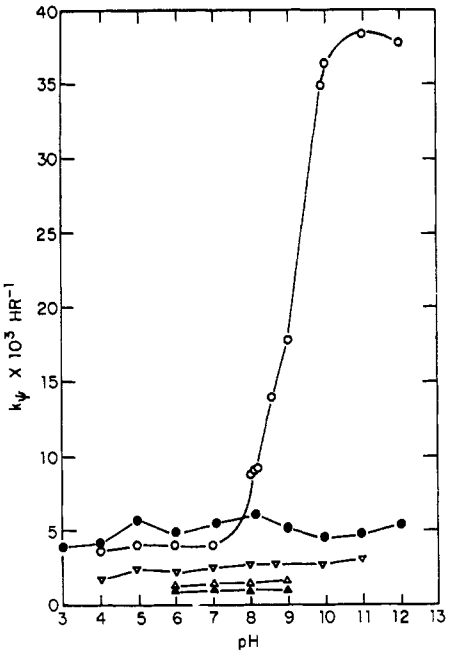


FIGURE 1: Plot of the observed pseudo-first-order rate constants (k_ψ) of tritium exchange at C-8 of purines as function of pH (from data of Table I). ○, Guanosine; ●, 1-methylguanosine; ▽, adenosine; △, guanine residues in native DNA; ▲, guanine residues in denatured DNA.

TABLE I: Observed Pseudo-First-Order Rate Constants (k_ψ) of Tritium Exchange at C-8 of Purine Derivatives at Various pH Values (37°).

	k_ψ ($\times 10^3$ hr^{-1})				
pH	Guanosine	1-Methylguanosine	Adenosine	Guanine Residues in Native DNA	Guanine Residues in Denatured DNA
3.0		4.02			
4.0	3.86	4.29	1.54		
5.0	4.06	5.40	2.43		
6.0	4.04	4.80	2.34	0.81	1.07
7.1	4.15	5.34	2.59	0.81	1.27
8.0	8.60		2.71	0.84	1.32
8.1	8.87	5.94			
8.15	9.05				
8.6	14.0		2.70		
9.0	18.4	4.91		0.76	1.43
9.9	35.0		2.69		
10.0	36.2	4.43			
11.0	38.5	4.57	3.18		
11.9		5.34			
12.0	37.75				

water solution at 100° for 7 hr to effect complete exchange (Shelton and Clark, 1967); the solution was then distilled and counted as above. The assay data indicated pseudo-first-order kinetics in all cases and the pseudo-first-order rate constants (k_ψ's) were calculated by a CDC 6600 computer using the least-squares approximation program. Reproducibility of the rate constants from run to run was usually approximately ±10% except for pH 11 and higher and for 1-methylguanosine (±20%). The following buffers were used unless otherwise noted: pH 3, 0.05 M citric acid–Na citrate; pH 4–5, 0.05 M acetic acid–Na acetate; pH 6–8.1, 0.05 M NaH_2PO_4–Na_2HPO_4; pH 8.6–9, 0.05 M Tris–Tris-HCl; pH 10, 0.05 M $NaHCO_3$–Na_2CO_3; pH 11–12, 0.05 M Na_2HPO_4–Na_3PO_4. The ionic strength was adjusted to 0.1 by addition of calculated amounts of solid NaCl. pH was measured on a Beckmann Zeromatic instrument at 37°. The pH of reaction mixtures were routinely checked after the incubation period and were found to have remained within ±0.05 pH unit of the initial value.

MEASUREMENT OF RADIOACTIVITY. A Nuclear-Chicago Mark I liquid scintillation counter was used. [^{3}H]H_2O was counted in Bray's solution (2.0 ml water in 20 ml) with an efficiency of 10.4%.

DETERMINATION OF pK_a's AT 37°. The spectrophotometric method of Shugar and Fox (1952) was used for determining pK_{a_1} and pK_{a_2} of guanosine and the pK_a's of 1-methylguanosine and 7-methylguanosine, all at 37°. Absorbances and pH values were measured at 37°, the former by using a Gilford Model 240 spectrophotometer equipped with an internal temperature probe system. The ionic strength of the buffers used was adjusted to 0.1 by addition of NaCl with the exceptions noted below. The following buffers were used: pH 0–2.5, HCl solutions; pH 3–5, 0.05 M acetic acid–Na acetate; pH 7–9.5, 0.05 M Tris–Tris-HCl; pH 10, 0.05 M $NaHCO_3$–Na_2CO_3; pH 11–12, 0.05 M NaH_2PO_4. In the case of the pH < 1 HCl solutions the ionic strength was not controlled. This represented only two points on the curves for the pK_{a_1}'s of guanosine and 1-methylguanosine. The following wavelengths were used for the absorbance measurements: 252 mμ: guanosine (pK_{a_1}), 1-methylguanosine; 250 mμ: guanosine (pK_{a_2}); 258 mμ: 7-methylguanosine.

Results

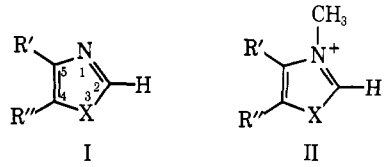
The experimental work of this investigation consisted of determining the kinetics of the exchange reaction in guanosine, 1-methylguanosine, adenosine, and in the guanine residues of DNA, under various conditions. At constant pH pseudo-first-order kinetics were observed in all cases. The effect of pH on the magnitude of the pseudo-first-order rate constants was studied extensively and the results are seen in Table I and Figure 1. The lack of effect of buffer concentration is illustrated in Table II. Addition of $CuCl_2$ (10^{-2} M or 1 M) had no effect on the exchange rate of guanosine.

TABLE II: Effect of Buffer Salt Concentration on the Rate of Tritium Exchange.

Buffer	Molarity (M)	k_ψ (10^3 hr^{-1}) Guanosine	Adenosine
Na acetate–acetic acid, pH 5.1	0.05	4.40	2.41
	0.20	4.10	1.95
Na_3PO_4–Na_2HPO_4, pH 11.5	0.05	42.0	3.4
	0.20	33.0	5.0

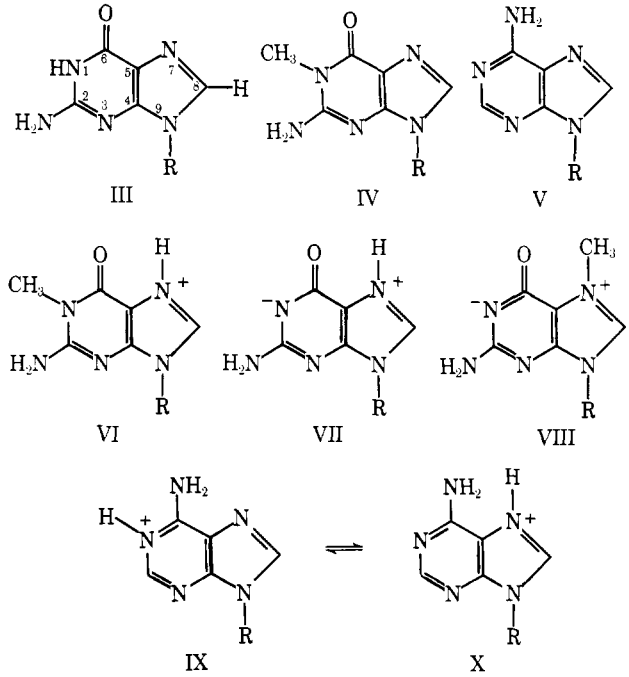
Discussion

We have been guided in our interpretation of the experimental data by the large body of work reported in the literature concerning an analogous system: the isotope exchange of the C-2 proton in five-membered heterocycles of type I, namely imidazole (a), thiazole (b), and oxazole (c) derivatives.

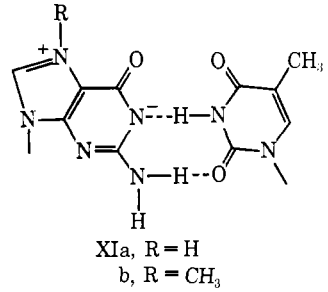


I, IIa, X = NR
b, X = S
c, X = O

The great interest in this particular class of proton-exchange reactions stems largely from its mechanistic relationship to the coenzyme action of thiamine (Breslow, 1958). As a result the mechanism of the exchange at C-2 in the models as well as in thiamine itself is now well understood. The structural similarity of the C-8 proton of purine nucleosides (III, V)



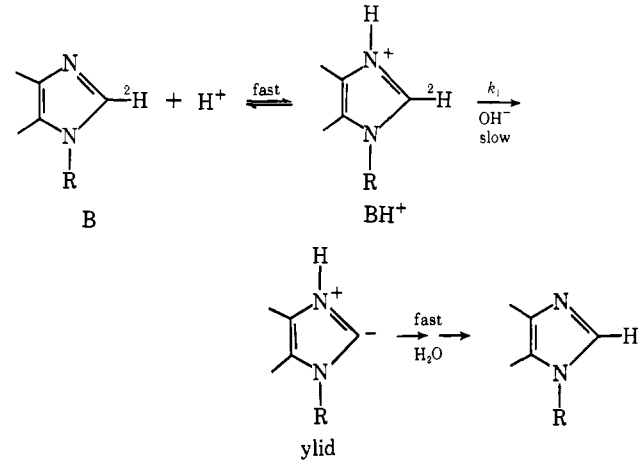
III–X: R = β-D-ribosyl



XIa, R = H
b, R = CH_3

to the C-2 proton of imidazoles (Ia) suggests an analogous mechanism of exchange. Considering the much greater complexity of the π-electron system of the purines, however, a different mechanism cannot be *a priori* excluded. As will be seen below the exchange mechanism of the model system I proved to be highly relevant indeed to our problem. Thus for clarity of discussion we shall first review the case of the simple five-membered heterocycles I, based on the work of several investigators (Harris and Randall, 1965; Coburn *et al.*, 1970; Vaughan *et al.*, 1970).

SCHEME I



Isotopic deuterium exchange at C-2 of compounds I occurs by the following mechanism (Scheme I). The prerequisite for the exchange at C-2 is protonation of the most basic heteroatom of the ring, N-1 (BH^+). Abstraction of the deuterium ion by a OH^- ion constitutes the next, rate-determining step resulting in an ylid type intermediate. The ylid is then quickly reprotonated by H_2O. The rate expression for this scheme is

$$\text{rate} = k_1[BH^+][OH^-] \qquad (1)$$

At constant pH this expression takes a pseudo-first-order form: rate = $k_\psi[B^0]$, where k_ψ is the pseudo-first-order rate constant and $[B^0]$ represents overall reactant concentration, regardless of ionization state. It can be derived readily that k_ψ is the function of $[H^+]$ by the following relationship

$$k_\psi = \frac{k_1 K_w}{K_a + [H^+]} \qquad (2)$$

where K_a is the dissociation constant of the protonated base BH^+. Equation 2 predicts that at $[H^+] \ll K_a$, k_ψ is independent of pH.

Proof for this mechanism comes from several sides. (a) The experimentally determined relationship between k_ψ and $[H^+]$ is in excellent agreement with the theoretical curve calculated from eq 2. (b) The *N*-1-alkyl derivatives (II) which may be regarded as analogs of the *N*-1-protonated cations display extremely fast isotope exchange at C-2: half-lives are of the order of a few minutes at neutral pH and room temperature (Breslow, 1958; Olofson *et al.*, 1964; Hafferl *et al.*, 1963; Haake *et al.*, 1969). In several cases direct comparison was made between the second-order rate constants (k_1 in eq 1) of the parent compound (I) and its *N*-alkyl derivative (II) and the values were found to be in good agreement (*e.g.*, Vaughan *et al.*, 1970). (c) Molecular orbital calculations indicated that the ylid is the most likely intermediate in the reaction (same reference).

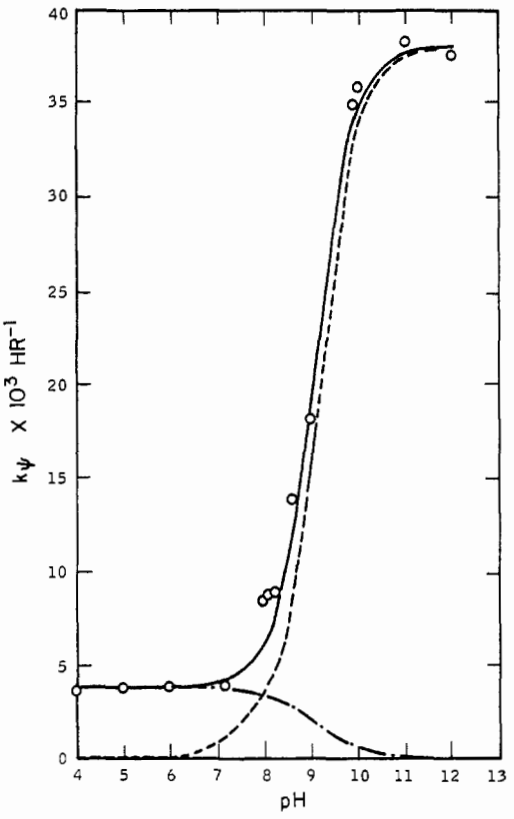


FIGURE 2: Comparison of the experimental and theoretical curves of k_ψ for the tritium exchange reaction of guanosine. O, Experimental points of k_ψ; ——, theoretical curve of k_ψ; ·—·, $k_\psi^{(1)}$ component of the theoretical k_ψ (see eq4 and 5); – –, $k_\psi^{(2)}$ component of the theoretical k_ψ.

Mechanism of the Tritium Exchange at C-8 of Purine Nucleosides. 1-METHYLGUANOSINE (IV). The rate of the C-8 tritium exchange follows pseudo-first-order kinetics. The pseudo-first-order rate constant k_ψ exhibits a striking independence of pH (Figure 1, Table I). This kinetics is fully explained by rate law 1 as applied to the present case: rate = k_1[VI][OH$^-$], because by substituting K_a of IV = 5.13×10^{-3} (Table III) into 2 the calculated k_ψ is rendered independent of pH in the entire pH range of our measurements (pH 4–11; [H$^+$] $\ll K_a$). Consequently, the kinetic data are consistent with the same "ylid" mechanism as that occurring in the case of the simpler models (Scheme I). The 7-protonated form of 1-methylguanosine (VI) (BH$^+$) is the kinetically active species. Rate-determining removal of tritium from C-8 by a OH$^-$ ion gives the ylid intermediate which is then reprotonated by the medium. Very recently direct evidence that the removal of tritium is rate limiting was reported in the case of two related systems, purine and adenine (Elvidge *et al.*, 1971).

GUANOSINE (III). The presence of an acidic hydrogen at N-1 in guanosine alters the kinetic picture so simple in the previous case where that function was blocked. This is evident from the fact that in the pH range of the N-1-H dissociation (pK_{a_2} = 9.13) the rate of exchange at C-8 undergoes an approximate tenfold increase with increasing pH (Figure 1, Table I). We postulate the following rate law to account for the observed kinetics:

$$\text{rate} = k_\psi[G^0] = \underbrace{k_1[HGH^+][OH^-]}_{\text{path 1}} + \underbrace{k_2[HG][OH^-]}_{\text{path 2}} \quad (3)$$

TABLE III: Values of Various Apparent K_a's at 37° and Ionic Strength of 0.1.[a]

K_{a_1} (III)	6.46×10^{-3}
K_{a_2} (III)	7.41×10^{-10}
K_a (IV)	5.13×10^{-3}
K_a (7-methylguanosine)	1.23×10^{-7}

[a] See Methods for details of determination.

where [G^0] represents overall guanosine concentration regardless of ionization state; HGH$^+$ is 7-protonated guanosine, HG is neutral guanosine. This rate law defines *two* pathways for the reaction, both of pseudo first order at constant pH. Thus it follows that k_ψ is the sum of the two pseudo-first-order rate constants $k_\psi^{(1)}$ and $k_\psi^{(2)}$ (corresponding to paths 1 and 2, respectively)

$$k_\psi = k_\psi^{(1)} + k_\psi^{(2)} \quad (4)$$

The expression of k_ψ as function of [H$^+$] is obtained readily by substituting [HG] + [HGH] + [G$^-$] for [G^0], $K_w/$[H$^+$] for [OH$^-$], K_{a_2}[HG]/[H$^+$] for [G$^-$], and [HG][H$^+$]/K_{a_1} for [HGH$^+$] into eq 3 (G$^-$ is symbol for guanosine-N-1 anion):

$$k_\psi = k_1 \frac{\frac{K_w}{K_{a_1}}}{1 + \frac{[H^+]}{K_{a_1}} + \frac{K_{a_2}}{[H^+]}} + k_2 \frac{\frac{K_w}{[H^+]}}{1 + \frac{[H^+]}{K_{a_1}} + \frac{K_{a_2}}{[H^+]}} \quad (5)$$

where K_{a_1} and K_{a_2} represent the two dissociation constants of guanosine (Table III) and the two right-hand terms are equal to $k_\psi^{(1)}$ and $k_\psi^{(2)}$, respectively. Given the expression we were now able to test the validity of the postulated rate law 3 by a common technique (*e.g.*, Harris and Randall, 1965): comparison of the theoretical curve of k_ψ as function of pH with the curve obtained experimentally. Values of the second-order-rate constants k_1 and k_2 necessary for calculating the theoretical curve from expression 5 were determined semiempirically as follows:

It can be shown[1] that at low pH (4–6) $k_\psi = k_\psi^{(1)}$. Thus, substitution of the pH 5 value of k_ψ (Table I) into expression 5 gives after omission of negligibly small terms $4.06 \times 10^{-3} = k_1(K_w/K_{a_1})$ and $k_1 = 1.07 \times 10^9$ M^{-1} hr^{-1}. Similarly, in the limiting region at high pH (11–12) $k_\psi \cong k_\psi^{(2)}$ and substitution of the pH 11 value of k_ψ into 5 gives $38.5 \times 10^{-3} = k_2(K_w/K_{a_2})$ and $k_2 = 1.16 \times 10^3$ M^{-1} hr^{-1}. Having thus determined k_1 and k_2 we could construct the theoretical curve of k_ψ in the entire pH range as shown in Figure 2. (The two individual components $k_\psi^{(1)}$ and $k_\psi^{(2)}$ are also indicated.) As seen the experimental points and the theoretical curve are in good agreement, indicating the validity of rate law 3.

As to the mechanistic interpretation of this kinetics the first term (path 1) indicates no doubt the same mechanism as that operating in 1-methylguanosine: base-catalyzed removal of the C-8 proton from the 7-protonated form (Scheme II, path 1). The interpretation of the second term of 3 (path 2) was less obvious: OH$^-$-catalyzed removal of the C-8 proton from *neutral* guanosine, although consistent with the rate law, seemed unlikely, especially in view of the apparent nonexistence of such a mechanism in the case of 1-methyl-

guanosine and the group of model compounds. We propose a more likely mechanism: the reactive species is not neutral guanosine but rather its kinetic equivalent, the *zwitterion* (VII; Scheme II, path 2). This form has a proton at the 7 position and thus abstraction of its C-8 proton is mechanistically analogous to path 1. No such form is possible, of course, in the case of 1-methylguanosine and adenosine. Kinetically the guanosine zwitterion ($GH^{\pm}$; *cf.* Scheme II) is equivalent with guanosine (HG) because they are in tautomeric equilibrium, independent of pH

$$[GH^{\pm}]/[HG] = K_{zw} \quad (6)$$

where K_{zw} represents the tautomeric equilibrium constant. Consequently, we may rewrite the rate expression 3 as

$$\text{rate} = k_{\psi}[G^0] = k_1[HGH^+][OH^-] + k_2'[GH^{\pm}][OH^-] \quad (7)$$

where

$$k_2' = k_2/K_{zw} \quad (8)$$

and it represents the second-order rate constant of the "zwitterion pathway" for the exchange reaction (path 2 in Scheme II). Having calculated the values of k_1 and k_2 (see above) it now remained to calculate k_2' in order to compare quantitatively the second-order rate constants of paths 1 and 2 (k_1 and k_2'). The calculation required knowledge of the value of K_{zw}, however (*cf.* eq 8). We were able to determine K_{zw} in the following way:

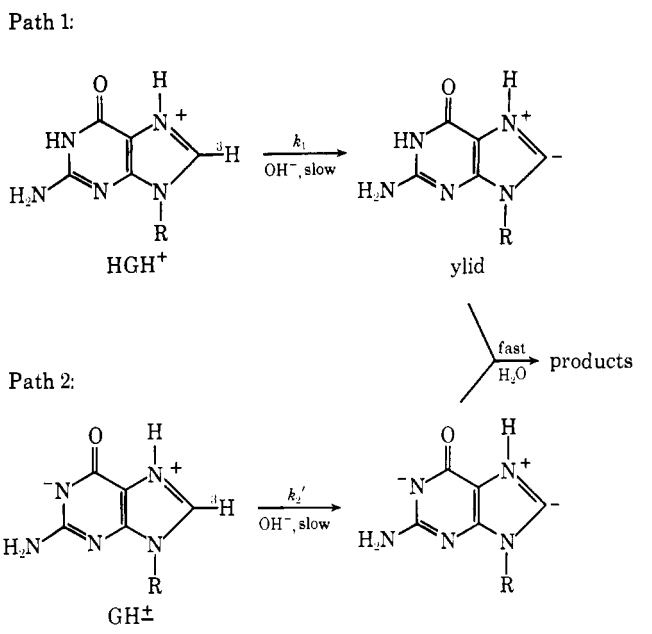
Calculation of K_{zw}. Tucker and Irwin (1951) developed a method for calculating tautomeric equilibrium constants, suitable for systems in which one tautomer greatly predominates. While they applied it to 4-hydroxyquinoline, the method is quite general and several authors have adapted it for nucleotide tautomerism studies (Kenner *et al.*, 1955; Katritzky and Waring, 1962; Wolfenden, 1969). The principle is the following: if the major tautomer and the methylated analog of the minor one form cations of similar structure on protonation, the ratio of their K_a values is equal to the tautomeric equilibrium constant. In our case the appropriate methylated analog of the zwitterion tautomer of guanosine ($GH^{\pm}$, VII) is 7-methylguanosine (VIIII). On protonation guanosine (III) and VIII give cations of similar structure as indicated experimentally by the similarity of their ultraviolet spectra (Jones and Robins, 1963). It then follows that $K_{zw} = K_{a(VIII)}/K_{a_1(III)} = 1.9 \times 10^{-5}$.

Having thus determined K_{zw} we then proceeded to calculate k_2' from 8 obtaining $k_2' = 6.0 \times 10^7\ M^{-1}\ hr^{-1}$.

Comparison of the values of the second-order rate constants of path 1 and path 2 (k_1 and k_2') reveals that k_1 is 17.5 times greater. This result is consistent with the expectation that in the zwitterion the positive charge of the imidazole ring should be somewhat decreased due to partial neutralization by the negative charge of the 6-membered ring and consequently abstraction of the C-8 proton should be less facile from the zwitterion than from the positive ion HGH^+.

In conclusion of the discussion on 1-methylguanosine and guanosine we wish to present a last, independent argument in support of the proposed ylid mechanism. The N-7 alkyl cations 7-methylguanosine (VIII) and 1,7-dimethylguanosine were found to display extremely fast exchange rates (Tomasz, 1970a; Broom and Robins, 1964) in full analogy to the model ylid type exchange systems I and II.

SCHEME II



ADENOSINE (V). The pseudo-first-order kinetics of the exchange show the same characteristic independence of pH as in the case of 1-methylguanosine (Figure 1, Table I); the value of k_{ψ} is approximately fivefold lower. A slower rate of exchange relative to guanosine and lack of acceleration by alkali has been noted earlier (Shelton and Clark, 1967).

The data suggest the same ylid type mechanism as that occurring in the guanosines: OH^--catalyzed abstraction of the C-8 proton from the 7-protonated form. The picture, however, is somewhat more complicated since the predominant site of protonation of adenosine is not N-7 (X) but rather N-1 (IX) (Jardetzky and Jardetzky, 1960; Jones and Robins, 1963). Yet we believe that the observed exchange occurs *via* the 7-protonated tautomer X presumably present to a small extent in equilibrium with IX. The latter form is discounted as the exchange active species not only because the ylid mechanism is not applicable but also because the kinetic results are incompatible with it: substitution into 2 of the K_a of adenosine (3.65; Wolfenden, 1969) which corresponds approximately to the acidity of the predominant 1-protonated form (IX) would predict a steep rise of k_{ψ} between 4 and 5, leveling off only near pH 6; the experimental curve (Figure 1) does not show such effect. An additional, strong argument against IX is the fact that its quaternary analog 1-methyladenosine hydrochloride does not display a fast rate of exchange (M. Tomasz, 1971, unpublished data). Let us then consider the alternative possibility that X is the exchange-active form.

Although the above cited investigations of protonated adenosine in solution identified N-1 as the *main site* of protonation the spectroscopic methods used do not rule out the presence of minor closely related tautomers such as X since they would escape detection if present in less than a 1:10 ratio to the predominant form. A positive though indirect indication of the existence of X is the fact that methylation of adenosine with dimethyl sulfate yields 7-methyladenosine in a ratio of approximately 1:10 to 1-methyladenosine (Lawley and Brookes, 1964), indicating the considerable relative nucleophilicity of the N-7 group of adenosine. If the equilibrium ratio of X to IX is 1:10 or lower the

K_a of X must be higher than the K_a of adenosine by a factor of 10 or more. Substitution of such a higher K_a value ($\geq 10^{-2.65}$) of the hypothetical X into 2 results in a theoretical k_ψ–pH profile which flattens out between pH 4 and 5 with increasing pH because of the limiting condition $[H^+] \ll K_a$. The experimental curve (Figure 1) is in agreement with this prediction. We feel on the basis of these arguments that the 7-protonated form (X) is the most plausible mediator of the exchange reaction in adenosine and thus the mechanism is fully analogous to that of guanosines.

It would be interesting to determine the rates at lower pH in order to estimate the actual pK_a value from the curve. The slight decline of k_ψ going from pH 5 to 4 may be taken as indication that this pK_a is around 2 or 2.5. We did not carry out measurements below pH 4, however, because adenosine is expected to be unstable under the prolonged incubation at low pH required by the assay.

Lack of buffer catalysis. A characteristic feature of the exchange reaction in the model systems (I) is lack of catalysis by H_2O and buffer anions (Haake *et al.*, 1969; Kemp and O'Brien, 1970). Similarly, our results with adenosine and guanosine indicate (Table II) no appreciable catalysis by buffer anions; thus the purines parallel the behavior of the five-membered heterocycles (I).

In summary, we propose that our kinetic data are best interpreted in terms of the following mechanism: isotope exchange at the C-8 position of purine nucleosides occurs by abstraction of the C-8 proton by OH^- when the imidazole ring is protonated at N-7, *i.e.*, it is positively charged (Scheme I). In the case of guanosine this condition is met not only by the 7-protonated cation but also by a tautomer of the uncharged molecule: the guanosine zwitterion (VII); the kinetics are fully consistent with the participation of this species.

Effect of Cu^{2+} ions on the rate of exchange in guanosine. Guanine derivatives form chelates with Cu^{2+} ions in solution, presumably involving the N-7 position as a binding site (Eichhorn *et al.*, 1966; Tu and Friederich, 1968). Since such a bond to a positive ion may be regarded as analogous to protonation, an enhancement of the exchange rate of guanosine might be expected in the presence of Cu^{2+} ions. Our data show that this is not the case, even at considerably greater Cu^{2+} concentrations than reportedly needed for complete binding of guanosine (Tu and Friederich, 1968). In view of the lack of definite knowledge of the structure of the Cu^{2+}–guanosine complex in solution interpretation of this result is not possible at this stage.

Exchange Reaction in Guanine Residues of DNA. Several authors reported kinetic data of the exchange reaction occurring in DNA (Searcy, 1968; Ostermann *et al.*, 1966; Doppler-Bernardi and Felsenfeld, 1969). Interpreting such data is difficult, however, as pointed out by the last authors, because they reflect the sum of the exchange at the adenine and guanine residues. We were able to study the kinetics due to the *guanine residues alone*. This was accomplished by using a new technique: instead of measuring the incorporation of tritium into DNA nonselectively in tritiated water (the way all previous work was done) we studied the release of tritium from DNA labeled predominantly in the 8 position of guanine residues (83% of the total label in C-8 of guanine). Although the selectivity of the position of label was thus not absolute we can show that in our rate assay at least 94% of the released counts originate from guanine residues, by the following argument.

Doppler-Bernardi and Felsenfeld (1969) concluded that the rate of exchange in DNA-adenine was slower than in DNA-guanine since upon analysis a 3:1 ratio of labeled guanine to labeled adenine residues was found at the extent of approximately 30–50% of complete overall exchange in the DNA-purines. Since our rate assays were carried out at or *below* this extent the corresponding exchange ratio in our case must have been *at least* 3:1. Taking the label distribution of our DNA into account, it then follows that at least 94% of the total released counts were due to the exchange at the guanine residues and therefore they may be regarded as the selective measure of that reaction alone.

From the results (Table I, Figure 1) the following conclusions may be drawn. The reaction follows pseudo-first-order kinetics at the pH values tested as in model guanosine. The lack of influence of pH on the rate suggests the usual ylid mechanism: 7-protonated guanine residues are the reactive forms (as in path 1, Scheme II, for guanosine). In contrast to guanosine, however, no rate increase is observed at alkaline pH. Thus the "zwitterion" mechanism (path 2, Scheme II) is not manifested in DNA or at least not up to pH 9. This is consistent with the fact that the pK_{a_2} of guanine residues of DNA is considerably higher than that of guanosine (Beaven *et al.*, 1955), thus the titration curve-like rate increase observed with guanosine (Figure 1) should be shifted to higher pH values in DNA. Because of fear of serious degradation of DNA at pH 10 or above on prolonged incubation (up to several days, as required for the assay) we did not test this assumption experimentally.

The rate in DNA-guanine is approximately one-fourth of that in guanosine in its "path 1" region. This decrease in rate may be explained by the decreased basicity of the guanine residues of DNA (Beaven *et al.*, 1955) but more complex factors possibly play a role as well. For instance, Srinivasan and collaborators (Hendler *et al.*, 1970) suggested that the C-8 atom of 7-methylguanine is much less electron deficient when the base is incorporated in a polymer; this may apply to 7-protonated guanine as well, rendering the abstraction of the C-8 proton less facile.

The rates of exchange at guanines of native and denatured DNA are practically identical. This result is consistent with that of Ostermann *et al.* (1966) who found the same with respect to the overall exchange at purine residues.

The exchange reaction in DNA is of considerable practical interest since it lends itself to a method for labeling DNA *in vitro*. Our conclusions should help in selecting the optimal conditions for such labeling experiments.

Significance of the zwitterion tautomer of guanosine. In an important study Miles and his coworkers (1963) demonstrated by infrared and nuclear magnetic resonance spectroscopy that guanosine exists in the keto–amino form III as the main tautomer in solution. The zwitterion VII was one of the alternative tautomers considered and excluded by the evidence. No mention of this form elsewhere has come to our attention. Thus calculation of the tautomeric equilibrium constant K_{zw} for VII $\rightleftharpoons$ III is presented here apparently for the first time and it may be added to the list of the few other tautomeric constants of nucleotide derivatives calculated previously. The low value of K_{zw} ($= 1.9 \times 10^{-5}$) explains, of course, the lack of spectroscopic detection of VII. On comparison, it is remarkable, that K_{zw} is almost identical with the calculated tautomeric constants of the imino/amino forms of cytosine ($K = 2.0 \times 10^{-5}$; Kenner *et al.*, 1955) and adenosine ($K = 2.5 \times 10^{-5}$, Wolfenden, 1969). Watson and Crick (1953) have suggested that spontaneous mutations might occur when one of the bases reacts in an alternate form during replication of DNA so as to cause mispairing; usually the

imino forms of cytosine and adenine and the enol forms of thymine and guanine are considered as possibilities. It appears now, that the zwitterion of guanine may be considered along with these "rare tautomers" since, in principle, it can form a base pair with thymine by two hydrogen bonds (XIa). This pairing scheme is basically due to Lawley and Brookes (1961) who proposed it for 7-methylguanine residues (XIb).

It should be pointed out that the value of K_{zw} determined for guanosine might be quite different from the K_{zw} of guanine residues in DNA; the same is true for the tautomeric constants of cytosine and adenosine referred to above. The constants for DNA could only be calculated if the appropriate K_n's of the bases *in DNA* would be known. It is expected that K_{zw} should also vary with the ionic strength of the medium.

In summary, we presented kinetic and theoretical evidence for the existence of the guanosine zwitterion and hypothesize that this form may be considered as a "rare tautomer" capable of spontaneous mispairing in the course of DNA replication.

References

Beaven, G. H., Holiday, E. R., and Johnson, E. A. (1955), *in* The Nucleic Acids, Chargaff, E., and Davidson, J. N., Ed., New York, N. Y., Academic Press, p 493.

Breslow, R. (1958), *J. Amer. Chem. Soc. 80*, 3719.

Broom, A. D., and Robins, R. K. (1964), *J. Heterocycl. Chem. 1*, 110.

Broom, A. D., Townsend, L. B., Jones, J. W., and Robins, R. K. (1964), *Biochemistry 3*, 494.

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

Coburn, R. A., Landesberg, J. M., Kemp, D. S., and Olofson, R. A. (1970), *Tetrahedron 26*, 685.

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

Eichhorn, G. L., Clark, P., and Becker, E. D. (1966), *Biochemistry 5*, 245.

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

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

Haake, P., Bausher, L. P., and Miller, W. B. (1969), *J. Amer. Chem. Soc. 91*, 1113.

Hafferl, W., Lundin, R., and Ingraham, L. L. (1963), *Biochemistry 2*, 1298.

Harris, T. M., and Randall, J. C. (1965), *Chem. Ind. (London)*, 1728.

Hendler, S., Furer, E., and Srinivasan, P. R. (1970), *Biochemistry 9*, 4141.

Jardetzky, C. D., and Jardetzky, O. (1960), *J. Amer. Chem. Soc. 82*, 222.

Jones, J. W., and Robins, R. K. (1963), *J. Amer. Chem. Soc. 85*, 193.

Katritzky, A. R., and Waring, A. J. (1962), *J. Chem. Soc.*, 1540.

Kemp, D. S., and O'Brien, J. T. (1970), *J. Amer. Chem. Soc. 92*, 2554.

Kenner, G. W., Reese, C. B., and Todd, A. R. (1955), *J. Chem. Soc.*, 855.

Lawley, P. D., and Brookes, P. (1961), *Nature (London) 192*, 1081.

Lawley, P. D., and Brookes, P. (1964), *Biochem. J. 92*, 19C.

Marmur, J., and Ts'o, P. O. P. (1961), *Biochim. Biophys. Acta 51*, 32.

Miles, H. J., Howard, F. B., and Frazier, J. (1963), *Science 142*, 1458.

Olofson, R. A., Thompson, W. R., and Michelman, J. S. (1964), *J. Amer. Chem. Soc. 86*, 1865.

Ostermann, L. A., Adler, V. V., Bebelaschvily, R., Savothkina, L. P., and Varshavsky, Y. M. (1966), *Biokhimiya 31*, 398.

Schweizer, M. P., Chan, S. I., Helmkamp, G. K., and Ts'o, P. O. P. (1964), *J. Amer. Chem. Soc. 86*, 696.

Searcy, D. G. (1968), *Biochim. Biophys. Acta 166*, 360.

Shelton, K. R., and Clark, J. M. (1967), *Biochemistry 6*, 2735.

Shelton, K. R., and Clark, J. M. (1968), *Biochem. Biophys. Res. Commun. 33*, 850.

Shugar, D., and Fox, J. J. (1952), *Biochim. Biophys. Acta 9*, 199.

Tomasz, M. (1970a), *Biochim. Biophys. Acta 199*, 18.

Tomasz, M. (1970b), *Biochim. Biophys. Acta 213*, 288.

Tu, A. T., and Friederich, C. G. (1968), *Biochemistry 7*, 4367.

Tucker, G. F., and Irwin, J. L. (1951), *J. Amer. Chem. Soc. 73*, 1923.

Vaughan, J. D., Mughrabi, Z., and Wu, E. C. (1970), *J. Org. Chem. 35*, 1141.

Watson, J. D., and Crick, F. H. C. (1953), *Nature (London) 171*, 964.

Wolfenden, R. V. (1969), *J. Mol. Biol. 40*, 307.