Proton Transfer from Heterocyclic Compounds. Part IV.[1] Guanine, Guanosine, Hypoxanthines, and Inosine

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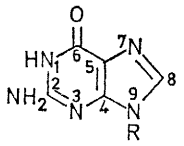
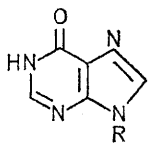
Rates of detritiation from C-8 of guanine, guanosine, hypoxanthine, 9-methylhypoxanthine, and inosine have been measured over a pH range at 85°. Two parallel rate-determining reactions are involved: one involves attack by hydroxide ion on the N-7 protonated substrates to form an ylide that is then reprotonated in a fast step; the other involves attack by hydroxide ion on the neutral substrates. This second mechanism becomes increasingly important at high pH (>9). The kinetic data provide no evidence for the involvement of a guanosine zwitterion.

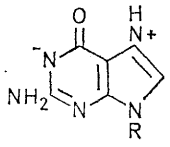
For various reasons [2] considerable interest has been expressed in studies of isotopic hydrogen exchange from heterocyclic compounds. With regard to the compounds under consideration in this paper Tomasz and her co-workers [3] have very recently measured the rates of detritiation from C-8 of guanosine [1] (1b), 1-methylguanosine, and guanine residues of DNA, over a pH range at



(1)
a; R = H
b; R = β-D-ribofuranosyl



(2)
a; R = H
b; R = β-D-ribofuranosyl
c; R = Me



(3)

37°. With the exception of the first compound which shows a rate increase in the pH range 7—11, the rates are virtually independent of pH. The reaction mechanism proposed is rate-determining hydroxide ion attack on the N-7 protonated substrates to form an ylide intermediate which is then reprotonated by the solvent in a fast step. In the case of guanosine an additional mechanism involving hydroxide ion attack, not on the neutral molecule but its kinetically indistinguishable zwitterion, was invoked. Support for the first mechanism came from the finding [4] that 7-methylguanosine catalyses the benzoin condensation. This compound exchanges 8-H immeasurably fast in D_2O at 28° as do 1,7-dimethylguanosine and 7-methylinosine,[4] a result that could have been anticipated in view of their close resemblance to the class of thiamine analogues which are well known [5] to undergo extremely rapid exchange at C-2. Several other qualitative observations of exchange of guanosine,[6-8] guanosine monophosphate [7,9,10] and RNA [9] (labelled by the *in vivo* incorporation of [8-^{3}H]guanosine) have been reported.

Bullock and Jardetsky [11] found that both hypoxanthine (2a) and inosine (2b) readily exchange 8-H for deuterium by heating in D_2O at 90—100° for 10—20 min. Subsequent studies [12-14] have shown that only for the 3-methyl derivatives of hypoxanthine, which form monocations by protonation at N-1, is the site of exchange (C-2) different from that of other purines (C-8). Recently Bergmann and his co-workers [15] have found the nuclear Overhauser effect useful in identifying the site at which isotopic hydrogen exchange occurs.

The compounds studied here differ from the benzimidazoles [16] and purines [1,17] previously reported upon in one important respect, namely that they all contain an NH group at C-1 of the pyrimidine nucleus. They are therefore good systems for investigating the effect of this group on rates of isotopic hydrogen exchange.

EXPERIMENTAL

Materials.—[8-^{3}H]Guanine sulphate (132 mCi mM^{-1}; solid), [8-^{3}H]guanosine (500 mCi mM^{-1}; aqueous solution), generally labelled [^{3}H]hypoxanthine (100 mCi mM^{-1}; solid), and [^{3}H]inosine (500 mCi mM^{-1}; aqueous solution) were from the Radiochemical Centre, Amersham. 9-Methylhypoxanthine (2c) was commercially available and [8-^{3}H]-9-methylhypoxanthine was prepared in the same way as the [2-^{3}H]benzimidazoles.[16]

Kinetics.—The methods used to follow the rates of detritiation are as given previously.[1,16,17] The percentage tritium at C-8 of hypoxanthine and inosine was determined as for

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[15] F. Bergmann, D. Lichtenberg, and I. Ringel, *J. Magnetic Resonance*, 1972, **6**, 600.
[16] J. A. Elvidge, J. R. Jones, C. O'Brien, E. A. Evans, and J. C. Turner, *J.C.S. Perkin II*, 1973, 432.
[17] J. A. Elvidge, J. R. Jones, C. O'Brien, E. A. Evans, and H. C. Sheppard, *J.C.S. Perkin II*, 1973, 1889.

adenosine.[1] For the first compound it was found to be 49% and for the second 80%.

RESULTS AND DISCUSSION

Guanine (1a), unlike any of the previously studied compounds, has 3 pK_a values in the pH range 0—14. These refer to protonation at N-7 and ionisation at N-1 and also N-9. The total concentration of guanine in a particular solution is given by equation (1) where

$$[B]_T = [BH_3^+] + [BH_2] + [BH^-] + [B^{2-}] \quad (1)$$

$[BH_3^+]$, $[BH_2]$, $[BH^-]$, and $[B^{2-}]$ represent the concentrations of guanine cation, neutral guanine, guanine monoanion, and guanine dianion respectively. The relevant dissociation constants are given by $K_a = [BH_2][H^+]/[BH_3^+]$, $K_a' = [BH^-][H^+]/[BH_2]$, and $K_a'' = [B^{2-}][H^+]/[BH^-]$ so that equation (1) takes the form (2).

$$[B]_T = [BH_3^+] + \frac{K_a[BH_3^+]}{[H^+]} + \frac{K_aK_a'[BH_3^+]}{[H^+]^2} + \frac{K_aK_a'K_a''[BH_3^+]}{[H^+]^3} \quad (2)$$

Rearranging equation (2) gives equation (3). By a similar procedure it can be shown that equation (4) holds. Assuming a rate equation of the form (5) we have equation (6). Hence equation (7) obtains.

$$[BH_3^+] = \frac{[B]_T}{1 + \frac{K_a}{[H^+]} + \frac{K_aK_a'}{[H^+]^2} + \frac{K_aK_a'K_a''}{[H^+]^3}} \quad (3)$$

$$[BH_2] = \frac{[B]_T}{1 + \frac{[H^+]}{K_a} + \frac{K_a'}{[H^+]} + \frac{K_a'K_a''}{[H^+]^2}} \quad (4)$$

$$\text{Rate} = k[BH_3^+][OH^-] + k'[BH_2][OH^-] \quad (5)$$

$$\text{Rate} = \frac{kK_w[B]_T}{[H^+] + K_a + \frac{K_aK_a'}{[H^+]} + \frac{K_aK_a'K_a''}{[H^+]^2}} + \frac{k'K_w[B]_T}{[H^+] + \frac{[H^+]^2}{K_a} + K_a' + \frac{K_a'K_a''}{[H^+]}} \quad (6)$$

Guanosine differs from guanine only by having a substituent at N-9. In the previous work on adenine and adenosine we have found that the ionisation of the ribose hydroxy-group need not be considered in assessing its effect on the rate of exchange from C-8. Assuming therefore that $K_a'' = 0$ equation (7) reduces to (8).

$$k_{obs} = \frac{kK_w}{[H^+] + K_a + \frac{K_aK_a'}{[H^+]} + \frac{K_aK_a'K_a''}{[H^+]^2}} + \frac{k'K_w}{[H^+] + \frac{[H^+]^2}{K_a} + K_a' + \frac{K_a'K_a''}{[H^+]}} \quad (7)$$

$$k_{obs} = \frac{kK_w}{[H^+] + K_a + \frac{K_aK_a'}{[H^+]}} + \frac{k'K_w}{[H^+] + \frac{[H^+]^2}{K_a} + K_a'} \quad (8)$$

For $K_a \gg [H^+] \gg K_a'$ and $k \gg k'$, $k_{obs} = kK_w/K_a$, corresponding to the pH region 3—7. Similarly for $[H^+] \ll K_a'$ (and hence $[H^+] \ll K_a$) and $k \gg k'$, $k_{obs} = k'K_w/K_a$, corresponding to the region pH >11.

The above treatment for guanine also holds for hypoxanthine; similarly the simplifications made for guanosine also apply for 9-methylhypoxanthine and inosine. The results (Table 1) when plotted (Figures 1 and 2) in terms of relative rate (defined as K_ak_{obs}/kK_w) illustrate the effect that is made by a group such as methyl or ribofuranosyl at N-9. The calculated lines are constructed by a trial and error procedure using equation (7) for guanine and hypoxanthine, and equation (8) for guanosine, 9-methylhypoxanthine, and inosine. The pK_a values that give the best fit (Table 2) compare well with the values obtained using the semi-empirical Perrin equation.[18] For example, the pK_a of 2·54 calculated for guanine at 85° compares with a value of 2·6 using equation (7).

Whilst the proposed rate equation accounts satisfactorily for the observed kinetics, there is the further possibility first mentioned by Tomasz and her coworkers,[3] that at high pH reaction involves the zwitterion (3), rather than the neutral substrate, and hydroxide

TABLE 1

Rate–pH data for [8-^{3}H]guanine (1a), [8-^{3}H]guanosine (1b), [8-^{3}H]hypoxanthine (2a), [8-^{3}H]inosine (2b), and [8-^{3}H]-9-methylhypoxanthine (2c)

Compound	(1a)	(2a)	(1b)	(2b)	(2c)
pH (at 85°)	$10^5k_{obs}/s^{-1}$				
0·51	0·025				
1·00	0·095			2·92	
1·50	0·23				
2·00	0·74				3·14
2·05			6·43		
2·15		1·80		7·25	
2·50	1·86				
2·75	2·71				
2·83	2·65				
3·00	3·15		13·5		
3·12		2·88		6·80	5·50
3·50	4·02		13·2		
4·07		2·95			
6·25	4·45	2·82	15·6	7·79	5·70
6·39				14·6	
6·96				36·9	
7·20		8·25			18·1
7·32				49·8	
7·65				73·6	
7·70				63·5	
7·75					37·8
8·02					45·5
8·38	7·70				
8·65			74·2		
8·77	8·90				
9·06	18·4				
9·17			118		
9·30	22·2				
9·48		11·7			
10·02	26·8				
10·10			151		
10·20		10·5			84·5
10·50	22·6	10·6	152	81·5	91·0
11·00	18·8				
11·20		6·86	158		93·4
11·40					88·5
11·50	12·1	3·56	146	84·0	89·0

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TABLE 2

Acidity constants and k' values for the various compounds

Compound	Literature values at 25°			From kinetics at 85°			
	pK_a	pK_a'	pK_a''	pK_a	pK_a'	pK_a''	k'/l mol^{-1} s^{-1}
Guanine	2·95 [a] *	9·32 [a] *	12·62 [a] *	2·6	8·6	11·2	0·18
Guanosine	1·90 [b]	9·25 [b]	12·33 [b]	1·7	8·7		0·95
Hypoxanthine	1·79 [b]	8·91 [b]	12·67 [b]	1·9	8·5	11·0	1·30
9-Methylhypoxanthine	1·83 [c] *	9·35 [c] *		1·9	8·8		4·5
Inosine	1·20 [d]	8·96 [b]	12·36 [b]	1·4	8·5		8·4

* At 20°.

[a] W. Pfleiderer, *Annalen*, 1961, **647**, 167. [b] J. J. Christensen, J. H. Rytting, and R. M. Izatt, *Biochemistry*, 1970, **9**, 4907. [c] D. J. Brown and S. F. Mason, *J. Chem. Soc.*, 1957, 682. [d] G. H. Beavan, E. R. Holiday, and E. A. Johnson, 'The Nucleic Acids,' eds. E. Chargraff and J. N. Davidson, Academic Press, New York, 1955, p. 493.

ion. The zwitterion has a positive charge on N-7 so that abstraction of tritium from C-8 would give rise to an ylide type intermediate and the process would be mechanistically analogous to the reaction pathway operating at low pH. Apparent support for the mechanism comes from the finding that 1-methylguanosine, which cannot exist as an analogous zwitterion, only undergoes exchange *via* the protonated molecule.[3] A similar situation is claimed to exist in the case of adenosine but more recent work [1] has shown that at high pH

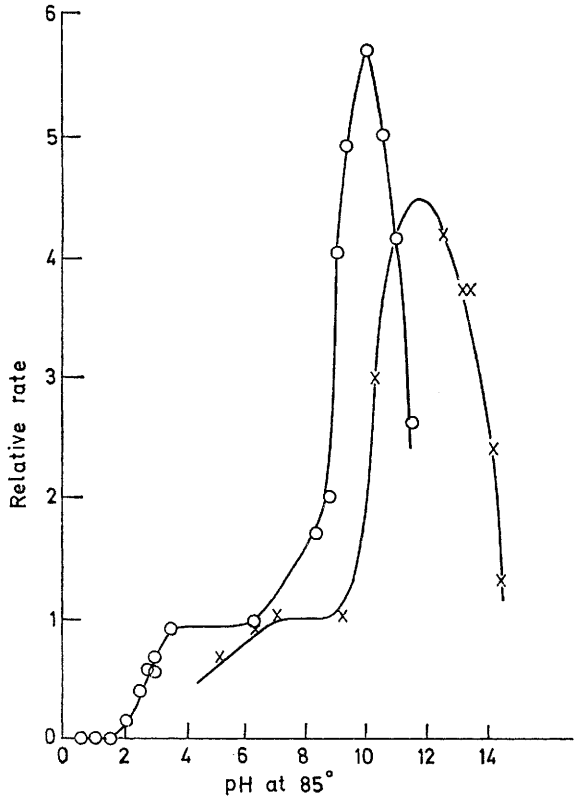

FIGURE 1 Rate–pH profile for [8-^{3}H]guanine (○) and hypoxanthine (×); points for the latter have been displaced by +3 pH units on the x axis

the rate of exchange of 8-H of adenosine increases dramatically. Furthermore the similar rate acceleration observed [17] for both 9-isopropyl- and 9-t-butyl-purines cannot be ascribed to the involvement of zwitterionic species as neither compound can exist as an appropriate

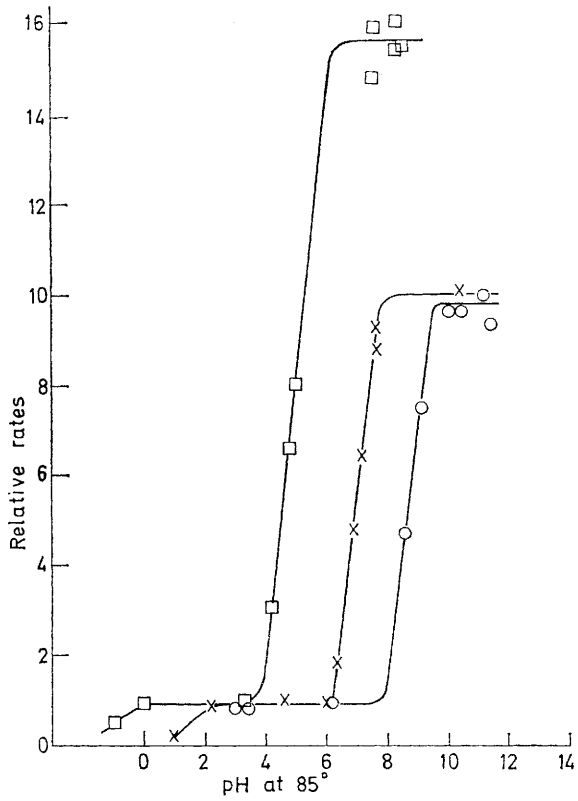

FIGURE 2 Rate–pH profile for [8-^{3}H]guanosine (○), [8-^{3}H]-inosine (×), and [8-^{3}H]-9-methylhypoxanthine (□); points for the latter have been displaced by −3 pH units on the x axis

zwitterion. We therefore favour a reaction mechanism at high pH that involves the neutral substrate and hydroxide ion; such a mechanism has been proposed for exchange at the 4(5)-position of imidazole [19] and the 2-position of thiazole,[20] pyridine,[21] and 4-alkylaminopyridines.[22]

We thank the Radiochemical Centre for supporting the work and the Director, Dr. W. P. Grove, for permission to publish the results.

[3/1817 *Received*, 3rd *September*, 1973]

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