Isotopic Hydrogen Exchange in Purines—Mechanisms and Applications

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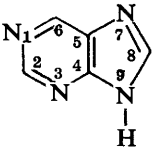
1 Introduction

The purines [derivatives of (1)] command important positions in both chemistry and biochemistry[1] and their versatility is reflected in the number of different roles they play in biological processes, from being one of the fundamental components of nucleic acids to being the basis of energy storage as in adenosine 5′-triphosphate. Furthermore, simple structural modifications of naturally occurring purines have made available a variety of purine analogues which are potent antagonists of many biological systems.



(1)

It is no great surprise, therefore, to find that deuterium- and tritium-labelled purines have found wide application in the biochemical sciences. Consequently, a better appreciation of all the factors that can influence the rates of deuterium or tritium loss will ensure that possible dangers of misinterpretation arising from the use of these labelled compounds will be minimized. Conversely, such studies can also lead to optimization of conditions for both labelling and storage of the appropriate compounds.

In contrast to other carbon acids that have been the subject of extensive

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[1] J. H. Lister, 'Purines', Vol. 24 (Part II) of 'The Chemistry of Heterocyclic Compounds', ed. D. J. Brown, Wiley-Interscience, New York, 1971.

investigation in recent years[2] and which undergo ionization as the uncharged species, the purines (like many other heterocyclic compounds) can exist in aqueous solution in a number of ionized forms, and therefore the number of available reaction pathways through which isotopic hydrogen exchange can occur is increased. Consequently, such studies can provide a convenient and often subtle way of obtaining information of both mechanistic and synthetic utility. Hydrogen isotope exchange in heterocyclic compounds[3] was first reviewed by one of us in 1974 and more recently Lister,[4] in dealing with some physicochemical aspects of purines, has considered both hydrogen exchange, radical reactions, and ionic alkylation reactions involving the C-8 position.

2 Historical Aspects

As long ago as 1952 Eidinoff *et al.*[5] reported on a biosynthetic method of incorporating tritium (from sodium [*methyl*-^{3}H]acetate) into yeast DNA, with subsequent hydrolysis leading to the isolation of labelled purine nucleosides and the corresponding bases. Although the position of the label was not identified, it was apparent that since the conditions used in the hydrolysis would lead to the removal of labile *N*- and *O*-bound tritium, the most likely sites were the C-8 positions of adenine and guanine. Eidinoff and Knoll[6] subsequently prepared tritiated and deuteriated adenine and guanine by heating the compounds with HTO or D_2O at 100 °C in the presence of a reduced platinum catalyst. In assigning the ^{1}H n.m.r. spectrum of purine, Ts'o and co-workers[7] found that purine exchanged its H-8 merely by heating in D_2O at 105 °C for 4 h, thereby dispensing with the need for an expensive platinum 'catalyst'. The product of this exchange reaction was shown to be identical to that obtained in the desulphurization of 8-mercaptopurine with deuteriated Raney nickel. Further confirmation of the site of exchange was provided by Bullock and Jardetzky[8] who unambiguously synthesized [8-^{2}H]purine by ring closure of 4,5-diaminopyrimidine with [$^2H_2$]-formic acid. In addition, hypoxanthine, inosine, adenine, adenosine, and 6-chloropurine were found to exchange H-8 by heating in D_2O at 100 °C for 10—20 min. Little or no exchange of H-2 occurred under these conditions.

Fox,[9] using a D_2O–DMF mixture, found that exchange occurred in adenosine, 6-chloropurine, and 7- and 9-benzyladenine at elevated temperatures. With 3-benzyladenine, however, both C-2 and C-8 deuteriation occurred, as is also the

[2] See for example (*a*) J. R. Jones, 'The Ionisation of Carbon Acids', Academic Press, London, 1973; (*b*) R. P. Bell, 'The Proton in Chemistry', 2nd edn., Chapman and Hall, London, 1973; (*c*) F. Hibbert in 'Comprehensive Chemical Kinetics', Elsevier, Amsterdam, Vol. 16, p. 97, 1976.
[3] J. A. Elvidge, J. R. Jones, C. O'Brien, E. A. Evans, and H. C. Sheppard, *Adv. Heterocycl. Chem.*, 1974, **16**, 1.
[4] J. H. Lister, *Adv. Heterocycl. Chem.*, 1979, **24**, 215.
[5] M. L. Eidinoff, H. C. Reilly, J. E. Knoll, and D. H. Marrian, *J. Biol. Chem.*, 1952, **199**, 511.
[6] M. L. Eidinoff and J. E. Knoll, *J. Am. Chem. Soc.*, 1953, **75**, 1992.
[7] M. P. Schweizer, S. I. Chan, G. K. Helmkamp, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, 1964, **86**, 696.
[8] F. J. Bullock and O. Jardetzky, *J. Org. Chem.*, 1964, **29**, 1988.
[9] J. R. Fox, Ph.D. Thesis, Univ. of Illinois, 1965.

case in 3-methylhypoxanthine.[10] More recently, Wong and Keck[11] have shown that the presence of an alkyl substituent at the 3-position, with concomitant localization of positive charge in the pyrimidine ring, causes an increase in the ratio of the H-2/H-8 exchange rates. In the detritiation[12] of [2,8-$^3H_2$]adenine under neutral conditions, exchange from the C-8 position was found to be *ca.* 2000 times faster than from the C-2 position.

As a preliminary study in the attempted tritiation of nucleic acids, McDonald and Philips[13] found that the H-8 of adenosine 5′-monophosphate exchanged in D_2O at 92 °C with a half-life of 90 min. Ostermann *et al.*[14] prepared tritiated nucleoside diphosphates by heating the unlabelled compounds in HTO at 100 °C, whilst the feasibility of labelling DNA *in vitro* was demonstrated by Fritzsche[15] who studied the deuteriation of adenosine and guanosine and the corresponding residues in DNA. Shelton and Clark[16] incorporated tritium into purine nucleotides by high temperature incubation in HTO and assessed their value as substrates in biochemical research. The tritium label was found to be stable for reasonable periods of time under physiological conditions, although for long-term reactions significant loss of label resulted, as confirmed by Evans *et al.*[17] [^{3}H]DNA prepared in this way has subsequently been used in hybridization reactions,[18,19] and Wechter[20] has used the same approach to label adenosine specifically in the presence of cytidine.

It seems somewhat surprising, therefore, that in view of the many studies that have been carried out, little or no attention has been given to the mechanistic details of these exchange reactions.

3 Experimental Methods

Although i.r.,[15,21] Raman,[22–24] and ^{1}H n.m.r.[7,11,25] spectroscopy have all been used to follow hydrogen isotope exchange in purines, we have found detritiation methods[26] to be the most useful. Because of the very low levels of radioactivity

[10] F. Bergmann and Z. Neiman, *Chem. Commun.*, 1969, 992.
[11] J. L. Wong and J. H. Keck, jun., *J. Chem. Soc., Chem. Commun.*, 1975, 125.
[12] J. A. Elvidge, J. R. Jones, C. O'Brien, and E. A. Evans, *Chem. Commun.*, 1971, 394.
[13] C. C. McDonald and W. D. Philips, *Biopolymers*, 1965, **3**, 609.
[14] L. A. Ostermann, V. V. Adler, R. Bibilashvily, and Ya. M. Varshavsky, *Biokhimiya*, 1966, **31**, 398.
[15] H. Fritzsche, *Biochim. Biophys. Acta*, 1967, **149**, 173.
[16] K. R. Shelton and J. M. Clark, jun., *Biochemistry*, 1967, **6**, 2735.
[17] E. A. Evans, H. C. Sheppard, and J. C. Turner, *J. Labelled Compd.*, 1970, **6**, 76.
[18] K. R. Shelton and J. M. Clark, jun., *Biochem. Biophys. Res. Commun.*, 1968, **33**, 850.
[19] D. G. Searcy, *Biochim. Biophys. Acta*, 1968, **166**, 360.
[20] W. Wechter, *Collect. Czech. Chem. Commun.*, 1970, **35**, 2003.
[21] M. Nakanishi, M. Tsuboi, and I. Nakagawa, *Bull. Chem. Soc. Jpn.*, 1976, **49**, 2011.
[22] G. J. Thomas jun., in 'Structure and Conformation of Nucleic Acids and Protein–Nucleic Acid Interactions', ed. M. Sundaralingam and S. T. Rao, University Park Press, Baltimore, Md., p. 253, 1975.
[23] (*a*) M. J. Lane and G. J. Thomas, jun., *Biochemistry*, 1979, **18**, 3839; (*b*) G. J. Thomas, jun. and M. J. Lane, *J. Raman Spectrosc.*, 1980, 9, 134.
[24] (*a*) J. Livramento and G. J. Thomas, jun., *J. Am. Chem. Soc.*, 1974, **96**, 6529; (*b*) G. J. Thomas jun and J. Livramento, *Biochemistry*, 1975, **14**, 5210.
[25] D. Lichtenberg and F. Bergmann, *J. Chem. Soc., Perkin Trans. 1*, 1973, 789.
[26] J. R. Jones, *Surv. Prog. Chem.*, 1973, **6**, 83.

that can be detected by liquid scintillation counting, the tritiated substrate needs to be present in solution only at very low concentration. Consequently, solubility problems rarely arise and measurements can be made in purely aqueous media. Furthermore, by measuring the increase in the radioactivity of the solvent (rather than the decrease in the radioactivity of the substrate) over the first 1—4% of the reaction, it has been possible to measure the rates of very slow reactions in a relatively short time interval (the so-called initial rate method). In this respect, the ready separation of the substrates from the solvent by freeze-drying is of great assistance. The pseudo-first-order detritiation rate constants (at 85 °C) are frequently of the order of 10^{-7}—10^{-5} s^{-1}, and being able to use the initial rate method is therefore a great advantage. The recent development of ^3H n.m.r. spectroscopy[27] means that a check on the specificity of labelling in the tritiated substrate can be made.

4 Rate–pH Profiles

The detritiation of [8-^{3}H]purine[28] or [8-^{3}H]adenine[29] over a pH range at 85 °C gives rise to a bell-shaped rate–pH profile (Figure 1, curve *a*). Although there have been one or two reports suggesting that general base catalysis occurs in reactions of this kind,[30] in all our studies overwhelming hydroxide-ion catalysis has been the rule. Under the experimental conditions purine and adenine can exist in one of three forms: (*i*) the neutral, BH; (*ii*) the protonated (either on N-1 or N-7), BH_2^+; or (*iii*) the anionic (ionization of N-9-H), B^-. Consequently, it would be reasonable to expect that the observed kinetics would conform to equation (1),

$$\text{Rate} = k[BH_2^+][OH^-] + k'[BH][OH^-] + k''[B^-][OH^-] \qquad (1)$$

so that a comparison between the relative reactivities of BH_2^+, BH, and B^- could be made. However, in practical terms, this possibility is governed by the values of the respective ionization constants:

[27] J. A. Elvidge, J. R. Jones, V. M. A. Chambers, and E. A. Evans, in 'Isotopes in Organic Chemistry', ed. E. Buncel and C. C. Lee, 1978, Vol. 4, p. 1.

[28] J. A. Elvidge, J. R. Jones, C. O'Brien, E. A. Evans, and H. C. Sheppard, *J. Chem. Soc., Perkin Trans. 2*, 1973, 1889.

[29] J. A. Elvidge, J. R. Jones, C. O'Brien, E. A. Evans, and H. C. Sheppard, *J. Chem. Soc., Perkin Trans. 2*, 1973, 2138.

[30] Maeda *et al.*[31a] claim to have observed catalysis by D_2O in $(CD_3)_2SO$–D_2O mixtures for exchange in adenosine, but we suggest that this result can be rationalized in terms of the changing $[D_2O]$ affecting $K_w(D_2O)$, which in turn partly governs the value of the pseudo-first order rate constant according to $k_\psi = k\,K_w/K_a$ (the rate of exchange in the pH-independent region of the rate–pH profile for adenosine[29,31a]).

More recently, Cohen and co-workers[31b] reported a 4.3-fold increase in the rate of C-2 deuteriation in 1-methylimidazole in the presence of 1 M acetate at pD 4.9. This result was obtained at a very sensitive position on the rate–pD profile for this substrate,[32] implying that the effect may, in part, be a consequence of pD or pK_a variations at the high salt concentration.

In our own studies in which we have utilized several imidazole and purine derivatives, including methylated guanosine and benzimidazole which model the protonated forms of these molecules, we have obtained no evidence for catalysis by bases other than hydroxide- or deuteroxide-ion. Clearly, more work is necessary in order to clear up this point.

[31] (*a*) M. Maeda, M. Saneyoshi, and Y. Kawazoe, *Chem. Pharm. Bull.*, 1971, **19**, 1641; (*b*) Y. Takeuchi, H. J. C. Yeh, K. L. Kirk, and L. A. Cohen, *J. Org. Chem.*, 1978, **43**, 3565.

[32] J. L. Wong and J. H. Keck, jun., *J. Org. Chem.*, 1974, **39**, 2398.

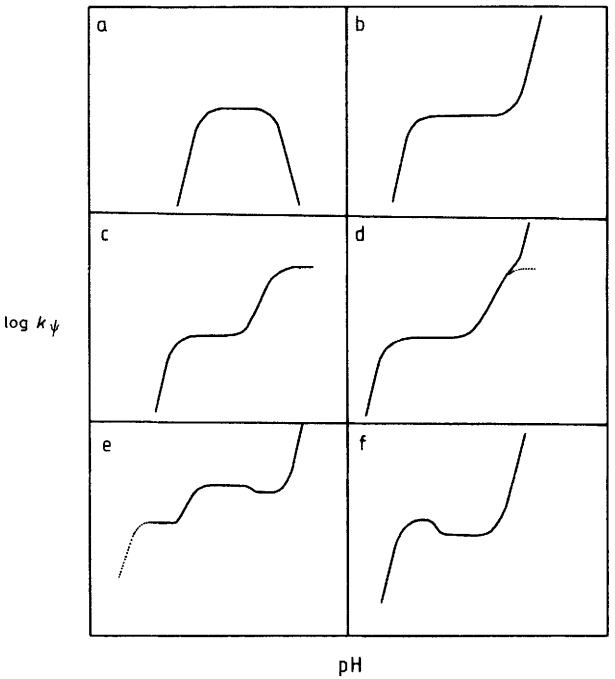


Figure 1 *Rate–pH profiles for isotopic hydrogen exchange from the* C-8 *position of* (*a*) *adenine*,[29] (*b*) 9-*Pr*^{i}-*purine*,[28] (*c*) *guanosine*,[36,41] (*d*) *theobromine*,[37] (*e*) *xanthosine*,[37] *and* (*f*) *adenosine 5′-monophosphate*[40]

K_a (= [BH] [H$^+$]/[BH$_2^+$]) and K_a' (= [B$^-$] [H$^+$]/[BH])

The observed rate–pH profile for purine and adenine is of the same form as that obtained for the detritiation of [2-^{3}H]benzimidazole;[33] by means of similar exchange studies on some chlorinated benzimidazoles and also on 1,3-dimethylbenzimidazolium bromide, we were able to show that the observed rate–pH profile is in accordance with equation (2).[33] Even at pH 12, where very little

$$\text{Rate} = k[BH_2^+]\,[OH^-] \qquad (2)$$

of the protonated form exists, reaction still proceeds by this mechanism; if the neutral form were reactive at such hydroxide-ion concentrations the rate would increase dramatically. In the rate–pH independent region, the effect of a decreasing concentration of protonated substrate is counteracted by the increasing concentration of hydroxide ions. This effect continues until the creation of a negative charge α to the site of exchange makes hydroxide-ion attack unfavourable on electrostatic grounds (*vide infra*), leading to a rate reduction. At very low pH, where the substrate is completely protonated, the decreasing hydroxide-ion concentration accounts for the fall-off in rate.[34]

[33] J. A. Elvidge, J. R. Jones, C. O'Brien, E. A. Evans, and J. C. Turner, *J. Chem. Soc., Perkin Trans. 2*, 1973, 432.

[34] Under these conditions ([H$^+$] $\gg$ K_a) the observed rate of exchange is directly proportional to [OH$^-$], the line, as demonstrated for imidazole,[35] passing through the origin.

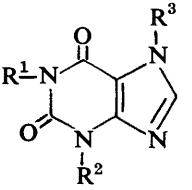
If we reduce the number of ionizable forms by, for example, blocking the N-9 position in purine, we see in the results for 9-Pr^{i}- and 9-Bu^{t}-purine[28] (Figure 1, curve *b*) how this affects the rate of exchange. At high pH there is a dramatic increase in rate due to the onset of a new mechanism involving hydroxide-ion attack on the neutral form, the appropriate rate equation being:

$$\text{Rate} = k[BH_2^+][OH^-] + k'[BH][OH^-] \qquad (3)$$

Adenosine[29] gives similar results to the 9-alkylpurines.

A logical extension of these studies would be to employ compounds containing an ionizable group in the pyrimidine ring, and this has been done for compounds such as guanosine and inosine[36] (Figure 1, curve *c*). In such cases the negative charge is developed at a site which is well-removed from that undergoing exchange, and the results suggest that had the studies been extended to still higher pH (> 11.5), hydroxide-ion attack on the anionic form of the substrate would have been observed, in accordance with equation (1).

The xanthines provide another example of how the presence of ionizable groups can influence the rates of exchange.[37–39] Here we can go from the trimethylated species [caffeine; (2), $R^1 = R^2 = R^3 = Me$] which can exist only in protonated (N-9) and neutral forms and which gives a rate–pH profile similar to adenosine,[37,38] to the three dimethylated species, theophylline [(2), $R^1 = R^2 = Me$,



(2)

$R^3 = H$], which gives a bell-shaped rate–pH profile, and theobromine [(2) $R^2 = R^3 = Me$, $R^1 = H$] and paraxanthine [(2), $R^1 = R^3 = Me$, $R^2 = H$], which additionally react *via* the anionic form at high pH (Figure 1, curve *d*). In general, the less the degree of methylation, the greater the number of potential mechanisms, so that in the case of xanthosine (9-β-D-ribofuranosylxanthine), the protonated, neutral, mono- and di-anionic forms all make a contribution to the overall rate[37] (Figure 1, curve *e*).

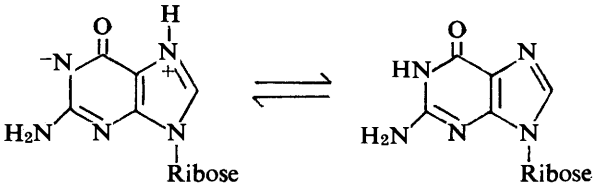
In the above examples, the protonation or ionization sites have been confined to either the pyrimidine or imidazole ring systems. There is the possibility that

[35] S. E. Taylor, Ph.D. Thesis, University of Surrey, Guildford, 1978.
[36] J. A. Elvidge, J. R. Jones, C. O'Brien, E. A. Evans, and H. C. Sheppard, *J. Chem. Soc., Perkin Trans. 2*, 1974, 174.
[37] J. R. Jones and S. E. Taylor, *J. Chem. Soc., Perkin Trans. 2*, 1979, 1253.
[38] M. Jelinska and J. Sobkowski, *Tetrahedron*, 1977, **33**, 803.
[39] M. Jelinska, J. Szydlowski, and J. Sobkowski, *Tetrahedron*, 1979, **35**, 663.

other groups may also influence the exchange, and this can be seen very clearly when we compare the rate–pH profiles for adenosine 5′-monophosphate[40] (Figure 1, curve *f*) and adenosine. An additional plateau region is observed in the pH range where ionization of the secondary phosphoric acid function occurs. That this is the most likely explanation is supported by the fact that no such second plateau is observed in the case of adenosine 3′,5′-cyclic monophosphate.[40]

5 Zwitterionic Contributions

It is possible that, in certain circumstances (*e.g.* at high pH), both the neutral form of the substrate and a kinetically equivalent species (a zwitterion) can both undergo exchange. Tomasz *et al.*[41] were the first to mention this possibility; in studies of isotopic hydrogen exchange at 37 °C from the C-8 position of guanosine, they observed a large rate acceleration at pH > 8, and this result, together with their failure to observe a similar acceleration in the case of 1-methylguanosine, was ascribed to the involvement of the guanosine zwitterion (3) rather than the neutral guanosine molecule (4). This idea is an attractive one as the zwitterion



(3) (4)

has a positive charge on N-7, and abstraction of tritium from C-8 would give rise to an ylide intermediate; the process is therefore analogous mechanistically to the reaction pathway operating at low pH. Clearly the involvement of this kinetically equivalent species cannot be discerned from the *shape* of the rate–pH profiles, but additional evidence is available.

Our own studies on adenosine[29] and on 9-Pr^{i}- and 9-Bu^{t}-purine,[28] which are unable to form zwitterions, showed large rate increases at high pH. More recent results on 1-methylinosine and 1-methylguanosine show the same trend.[42] A comparison of these results with those obtained[36] for inosine and guanosine serves to show that at low pH, where the effective mechanism is between hydroxide ion and the protonated species, methyl substitution at N-1 has but a marginal effect on the rate. In sharp contrast, the results at high pH show that N-1-methylation brings about a large rate retardation. Clearly in both guanosine and inosine the hydroxide ion reacts with the neutral (rate constant k') and zwitterionic (rate constant $k_{\pm}$) forms, and the observed second-order rate constant k_{obs} is given by [37,42]

[40] J. R. Jones and S. E. Taylor, *J. Chem. Soc., Perkin Trans. 2*, 1980, 441.
[41] M. Tomasz, J. Olsen, and C. M. Mercado, *Biochemistry*, 1972, **11**, 1235.
[42] J. R. Jones and S. E. Taylor, *J. Chem. Soc., Perkin 2*, 1979, 1587.

$$k_{obs} = k' + k_{\pm} K_{zw} \quad (4)$$

where $K_{zw} = [BH^{\pm}]/[BH]$. Kinetic evidence in support of the involvement of zwitterions has also been obtained for various xanthines,[37] nucleotides,[40] and histidine derivatives.[43] *X*-Ray crystallographic studies[44] show that even in the solid state the free acids of nucleotides exist as zwitterionic species.

6 Sites of Protonation

In formulating a mechanism for isotopic hydrogen exchange in purines, we assumed that the reactive species is the N-7-protonated form, present in all cases to some extent. However, the extensive non-kinetic investigations which have been carried out in an attempt to identify the site(s) of protonation do not always lead to consistent results. *X*-Ray crystallographic studies on purine show that the crystalline salt is protonated at N-7,[45] whereas N-1 is favoured for adenine[46] and adenosine.[47,48] Alkylation reactions invariably lead to a mixture of isomers[49,50] and from a study of the variation in ^{13}C—H coupling constants with pH, it has been concluded that, in solution, purine is partially protonated at N-1, N-3, and N-7.[51] On the basis of linear free energy relationships (LFERs), the results of our isotopic hydrogen exchange studies support the idea that protonation does not exclusively involve N-7 in most purines.[52] The accumulated mass of detritiation rate data in the literature which relates to azolium species can be roughly classified into two main groups, according to their protonation behaviour.[52] In the first group,[53a] data for the compounds imidazole, benzimidazole, 1,2,4-triazole, tetrazole, benzoxazole, benzothiazole, thiazole, and benzoselenazole, which are known to possess a single protonation site (N-3, equivalent to N-7 in purines) adjacent to the position of exchange, exhibit an excellent LFER between log k and pK_a (the solid line shown in Figure 2), with a slope of -0.72.[52,53a]

In contrast to this, the data for the purines, in which the protonation site is not as well defined, reveal that only in a few instances do the points lie on the line drawn for the azoles. We have interpreted this in terms of different degrees of N-7 protonation in the purines.[52] On this basis, the finding that the data for compounds 1—5 and 8 fall on the line (Figure 2), suggests their preference for exclusive N-7 protonation. Compounds 6, 7, 9—17, 23, and 24 appear to fall on a line having approximately the same slope as that derived from the azole data,

[43] J. A. Elvidge, J. R. Jones, R. Salih, M. Y. Shandala, and S. E. Taylor, *J. Chem. Soc., Perkin Trans. 2*, 1980, 447.
[44] M. Sundaralingam and P. Prusiner, *Nucleic Acids Res.*, 1978, **5**, 4375.
[45] D. G. Watson, R. M. Sweet, and R. Marsh, *Acta Crystallogr.*, 1965, **19**, 573.
[46] W. Cochran, *Acta Crystallogr.*, 1951, **4**, 81.
[47] J. Kraut and L. H. Jensen, *Acta Crystallogr.*, 1963, **16**, 17.
[48] M. Sundaralingam, *Acta Crystallogr.*, 1966, **21**, 495.
[49] B. C. Pal, *Biochemistry*, 1962, **1**, 558.
[50] P. D. Lawley and P. Brookes, *Biochem. J.*, 1964, **92**, 19c.
[51] J. M. Read, jun. and J. H. Goldstein, *J. Am. Chem. Soc.*, 1965, **87**, 3440.
[52] J. R. Jones and S. E. Taylor, *Tetrahedron Lett.*, 1981, in the press.
[53] (*a*) J. A. Elvidge, J. R. Jones, R. Salih, M. Y. Shandala, and S. E. Taylor, *J. Chem. Res.* 1980, (S) 172; (M) 2375. (*b*) D. Lichtenberg, F. Bergmann, and Z. Neiman, *J. Chem. Soc.(C)*, 1971, 1676. (*c*) Y. Maki, M. Suzuki, K. Kameyama, and M. Sako, *J. Chem. Soc., Chem. Commun.*, 1981, 658.

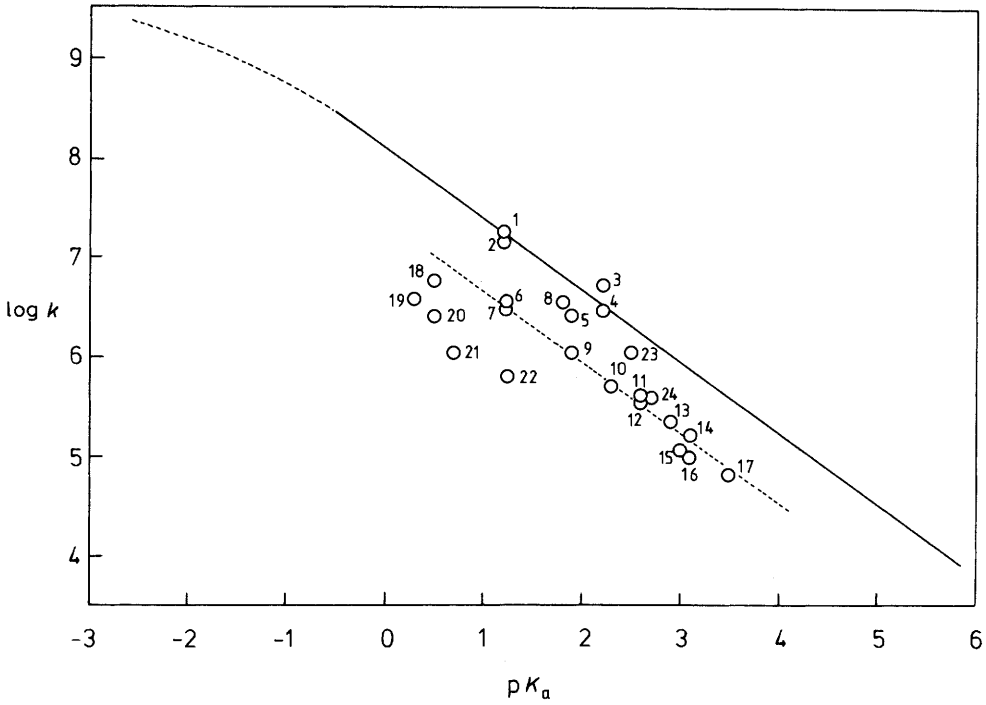


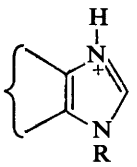
Figure 2 *Plot of log k* vs. pK_a *for the detritiation at the* C-2 *position of various azolcs (solid line, taken from ref.* 53*a) and the* C-8 *position in a number of purines. The numbers refer to the following compounds:* 1-*methylinosine* (1), *inosine* (2), 1-*methylguanosine* (3), *guanosine* (4), 9-*methylhypoxanthine* (5), 6-*mercapto-purine riboside* (6), 6-*mercaptopurine* (7), *xanthosine* (8), *hypoxanthine* (9), *purine* (10), *puromycin* (11), *guanine* (12), *adenosine* (13), *adenosine* 3′-*monophosphate* (14), *adenosine* 5′-*monophosphate* (15) *adenosine* 3′,5′-*cyclic monophosphate* (16), *adenine* (17), *paraxanthine* (18), *theobromine*, (19), *caffeine* (20), *theophylline* (21), *xanthine* (22), 9-Pr^i-*purine* (23), *and* 9-Bu^t-*purine* (24) (*taken from ref.* 52)

but which is displaced by 0.7 units on the log k axis, indicative of a lower (*ca.* 20%) degree of N-7 protonation. Points for the xanthines [compounds (18)—(22)] are displaced still further, in line with a previous suggestion[53b] that significant protonation occurs at O-6 as well as at N-9, making these substrates *ca.* 30-fold less reactive to exchange. Additional supportive evidence for the participation of varying degrees of N-7(9) protonation in purines is based on further LFER data,[53a] as well as a very recent paper[53c] in which pseudo-first-order deuteriation exchange rates were measured for a series of 9-substituted adenines, the highest rates being encountered with those derivatives which showed a greater tendency for N-7 over N-1 protonation (an interpretation not stressed by the authors).

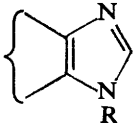
7 Metal-ion Effects

Recent studies by Kluger[54] and Cox[55] have drawn attention to the fact that, although the rates of ionization of carbon acids have been extensively investigated both as a function of acid strength and the basicity of the medium, very little attention has been paid to the way in which metal ions can influence the rates, even though in both hydrolysis and hydration reactions quite startling rate accelerations have been reported. Here again, the purines are excellent substrates for investigating such effects, as they are known[56] to complex very readily with a number of metal ions; however, little is known about how complex formation affects the reactivity.

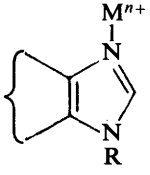
If, in the absence of zwitterionic contributions, the two reactive forms in isotopic hydrogen exchange from the C-8 position are (5) and (6) it can be seen that in the presence of added metal ions, species such as (7) will be formed, and



(5)



(6)



(7)

relative reactivities can be compared. This may be done either in a competitive manner (precipitation of metal ions at high pH rather limits this approach) or by preparing and isolating the appropriately labelled complex and studying its rate of exchange directly. In the one study where both of these approaches have been used, consistent results have been obtained.[57]

Detritiation of 1-methyl[8-^{3}H]inosine under conditions where the operative mechanism is between the protonated substrate and hydroxide ion shows that

[54] R. Kluger and P. Wasserstein, *J. Am. Chem. Soc.*, 1973, **95**, 1071.
[55] B. G. Cox, *J. Am. Chem. Soc.*, 1974, **96**, 6823.
[56] R. M. Izatt, J. J. Christensen, and J. H. Rytting, *Chem. Rev.*, 1971, **71**, 439.
[57] J. R. Jones and S. E. Taylor, *J. Chem. Soc., Perkin Trans. 2*, 1979, 1773.

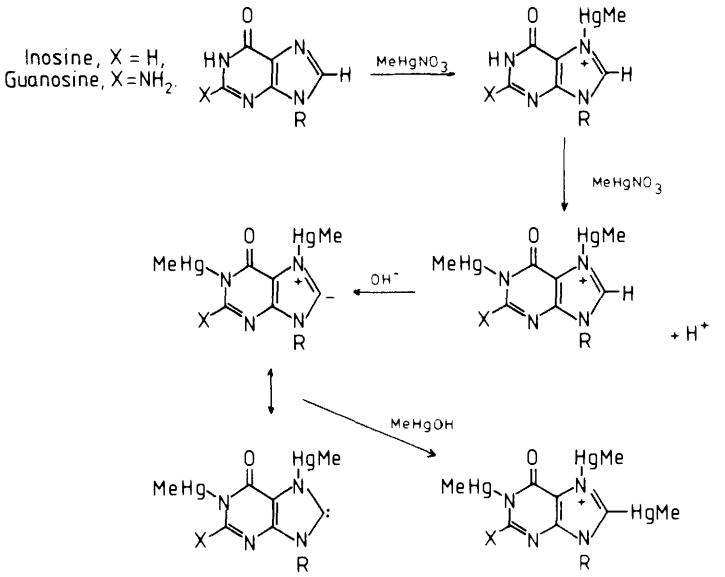
metal ions influence the rate in different ways: Zn^{II} ions have virtually no effect, whereas Ag^{I} ions retard the rate to a greater extent than do Cu^{II} ions. The metal-complexed species, although not as reactive as the protonated substrate, are *a.c* 10^4—10^6 times more reactive than the neutral form, and we have called this the 'metal activating factor' (**maf**), analogous to Stewart and Srinivasan's 'proton activating factor' (**paf**)[58] and our 'anion deactivating factor' (**adf**).[59] The second-order detritiation rate constant for the complex cation *cis*-[(1,2-diaminoethane)(guanosine)$_2$]Pt^{II} at 25 °C has a value[57] of 2.7 × 10^5 as compared to 6.1 × 10^5 l $mol^{-1}s^{-1}$ for 7-methylguanosine (8) which also has a positive charge located at the N-7 position.[42]

These results provide a rationale for the observation that co-ordination of heavy-metal ions to the N-7 position causes a rapid disappearance of the H-8 signal in the ^1H n.m.r. spectra of inosine and guanosine derivatives in D_2O,[60] as well as explaining the absence of rate accelerations when studies are carried out at low pH.[41] This metal activating effect has important synthetic consequences: witness the mercuriation of the C-8 position of purine nucleotides with $Hg(OAc)_2$ under mild conditions[61] and, perhaps more appropriately, the ready formation of C-8-bonded inosine and guanosine methylmercurials,[62] reactions which are believed to proceed by the route shown in Scheme 1. The same principles have recently been used to identify the histidine residues that act as metal-ion (Zn^{II}, Cu^{II}) binding sites in the metalloenzymes β-lactamase-II[63] and superoxide dismutase.[64] The latter study, in particular, provides an example of the application of our previous finding[65] of decreased rates of detritiation of [2-^{3}H]imidazole in the presence of metal ions, inasmuch as the decreased rate of deuteriation of certain histidine residues is ascribed to metal-ion binding.[64] Similar findings have recently been reported[66] for some Co^{III} complexes of imidazole.

8 Magnitude of Electrostatic Factors

The results in Table 1 show that protonation at the N-7 position of purines which are unable to exist as zwitterions consistently leads to rate accelerations (**pafs**) of the order of 10^7—10^9, *ca.* 10^2—10^3 greater than the effects observed with the few metal ions so far studied.[57] Only in the case of tetrazole (9), one of the strongest

[58] R. Stewart and R. Srinivasan, *Acc. Chem. Res.*, 1978, **11**, 271.
[59] S. E. Taylor, *Can. J. Chem.*, 1980, **58**, 86.
[60] (*a*) S. Mansy and R. S. Tobias, *J. Chem. Soc., Chem. Commun.*, 1974, 957; (*b*) S. Mansy and R. S. Tobias, *Biochemistry*, 1975, **14**, 2952; (*c*) G. Y. H. Chu and R. S. Tobias, *J. Am. Chem. Soc.*, 1976, **98**, 2641.
[61] R. M. K. Dale, D. C. Livingstone, and D. C. Ward, *Proc. Natl. Acad. Sci. USA*, 1973, **70**, 2238.
[62] (*a*) E. Buncel, A. R. Norris, W. J. Racz, and S. E. Taylor, *J. Chem. Soc., Chem. Commun.*, 1979, 562; (*b*) E. Buncel, A. R. Norris, W. J. Racz, and S. E. Taylor, *Inorg. Chem.*, 1981, **20**, 98.
[63] G. S. Baldwin, S. G. Waley, and E. P. Abraham, *Biochem. J.*, 1979, **179**, 459.
[64] (*a*) A. E. G. Cass, H. A. O. Hill, J. V. Bannister, W. H. Bannister, V. Hasemann, and J. T. Johansen, *Biochem. J.*, 1979, **183**, 127; (*b*) J. C. Dunbar, J. T. Johansen, A. E. G. Cass, and H. A. O. Hill, *Carlsberg Res. Commun.*, 1980, **45**, 349.
[65] D. H. Buisson, J. R. Jones, and S. E. Taylor, *J. Chem. Soc., Chem. Commun.*, 1975, 856.
[66] N. S. Rowan, C. B. Storm, and R. Rowan, *J. Inorg. Biochem.*, 1981, **14**, 59.



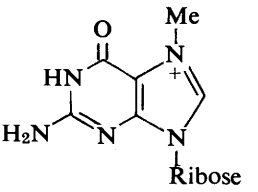
Scheme 1

Table 1 *Proton activating factors* (**pafs**), *anion deactivating factors* (**adfs**), *and metal activating factors* (**mafs**) *at* 85 °C *for some purines and azoles*

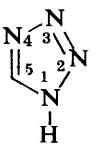
Compound	**paf**	**adf**	**maf**	*Refs.*
Adenosine	1.1×10^7			29, 58
9-Pr^i-purine	1.3×10^8			28, 58
9-Bu^t-purine	1.2×10^8			28, 58
Caffeine	9.3×10^7			37, 59
Theobromine[a]	5.9×10^7	7.2×10^{-2}		37, 59
Paraxanthine[a]	5.6×10^4	1.8×10^{-2}		37, 59
Guanosine[a]	5.1×10^5			36, 42
1-Methylguanosine	2.1×10^9		$6 \times 10^6(Cu^{II})$	42, 57
Inosine[a]	9.3×10^5			36, 42
1-Methylinosine	9.5×10^8		$1 \times 10^6(Cu^{II})$ $2 \times 10^4(Ag^I)$	42, 57
Thiazole	8.3×10^8			53*a*
Benzoxazole	1.4×10^8			53*a*
Tetrazole	6.3×10^7	2.3×10^{-7}		53*a*

[a] Potentially zwitterionic molecules.

heterocyclic carbon acids,[2] has it been possible to compare the effects of protonation and ionization at similar sites.[53a] Indeed, log(**adf**) for ionization at N-1 (−6.64) is very nearly equal and opposite to log(**paf**) for protonation at N-4



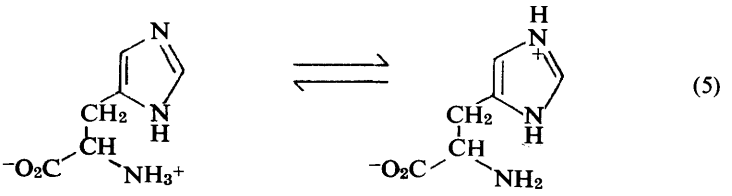
(8)



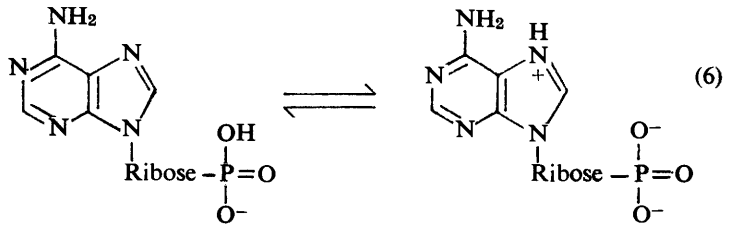
(9)

(7.79).[67] The latter value is subject to some uncertainty because the pK_a used in its calculation was for 1-methyltetrazole.[53a] These large effects, arising as they do from protonation or ionization at 'fixed' sites, contrast markedly with the very small electrostatic effects associated with charged groups in a more 'flexible' arrangement. Thus, a comparison of the data for histidine and histamine exchange[43] shows that the inhibiting effect of a negatively charged carboxylate group is small, as is also the case for a phosphate group in various nucleotides.[35,40] Similarly, in histidine derivatives when protonation occurs both in the imidazole ring and on the amino-group, the reactivity of the doubly protonated species is similar to that of singly protonated substrates of the same pK_a.[43] The main effect of these charged groups in the side-chain is to facilitate proton transfer *via* zwitterion formation, as shown in equations (5) and (6) for histidine and adenosine 5′-monophosphate, respectively.

In compounds capable of existing as zwitterions, *e.g.* guanosine, the measured



(5)



(6)

[67] It is unlikely that in solution the proton is localized at N-4; however, we are assuming that exchange from the N-4-protonated species will be kinetically dominant.

paf values are lower than for compounds unable to form such species (Table 1). This is because the observed second-order rate constant, k_{obs} is (as mentioned previously) a composite of two terms, k' and $k_{\pm}$ which refer to the neutral molecule and zwitterion, respectively [equation (4)]. In the case of those xanthines that react as the N-1 or N-3 anions (theobromine and paraxanthine, respectively), the corresponding **adf**s have been evaluated.[59] Consequently, as the zwitterions of these species differ from the protonated molecule only inasmuch as ionization has occurred at N-1, equation (7) holds. Using the derived value of $k_{\pm}$ in equation

$$k_{\pm} = k \times \mathbf{adf} \tag{7}$$

(4) it is now possible to calculate values of K_{zw}; experimentally, these values cannot be measured directly.[59] The **adf** values so far obtained (Table 1) for ionization at N-1 are as expected on electrostatic grounds, and are a good deal smaller than those resulting from ionization at a group immediately adjacent to that undergoing exchange.

9 Ionization Constants

In view of the importance of heterocyclic compounds and the fact that they can exist in various ionized forms in aqueous solution, a great deal of effort has been directed to the measurement of their various ionization constants. Isotopic hydrogen exchange studies such as ours provide a convenient route to such information. Thus, for compounds such as purine and adenine, which conform to equation (2) and give bell-shaped rate–pH profiles, it can be shown that the pseudo-first-order rate constant, k_{ψ}, is given by equation (8).

$$k_{\psi} = \frac{k\,K_w}{K_a + [H^+] + \dfrac{K_a K_a'}{[H^+]}} \tag{8}$$

$$K_a = [BH][H^+]/[BH_2^+] \text{ and } K_a' = [B^-]\,[H^+]/[BH]^{28,29,33}$$

In the pH–rate independent region, $k_{\psi} = k\,K_w/K_a$, so that if the rate at low pH is expressed relative to this value, equation (9) can be derived.[33]

$$pH = pK_a + \log_{10}[R/(1 - R)] \tag{9}$$

A plot of pH against $\log_{10}[R/(1 - R)]$ therefore provides a value of the pK_a. At high pH, when $K_a \gg K_a' > [H^+]$, equation (10) holds, such that inspection of the rate–pH profile at $R = 0.5$ gives the pK_a' value.

$$R = \frac{[H^+]}{[H^+] + K_a'} \tag{10}$$

For compounds that give rise to other kinds of rate–pH profiles it is still possible to obtain values of the ionization constants. In such cases, the rates are again expressed in relative terms and the best fit of a theoretical rate–pH profile to the experimental data provides the necessary information.

Most of the acidity constant data in the literature refer to a single temperature, usually 25 °C. Frequently the results are required at other temperatures.

As $-d(\Delta G^\circ)/dT = \Delta S^\circ$ and $\Delta G^\circ = 2.303RT\,pK_a$, equation (11) holds.[68] For

$$-\frac{d(pK_a)}{dT} = \frac{pK_a}{T} + \frac{\Delta S^\circ}{2.303RT} = \frac{pK_a + 0.218\Delta S^\circ}{T} \qquad (11)$$

equilibria of the kind $BH_2^+ \rightleftharpoons BH + H^+$, Perrin[68] was able to show that ΔS° values lie in the range -4 ± 6 cal $K^{-1}mol^{-1}$, so that equation (11) reduces to $-d(pK_a)/dT = (pK_a - 0.9)/T$. This equation, named after its author, has met with a good deal of success, and prompted us to look at equilibria of the kind $BH \rightleftharpoons B^- + H^+$ (ionization constant K_a').[69] Values of pK_a' at 85 °C for many compounds have been obtained from our isotope exchange experiments and, as equation (11) can be rearranged to give equation (12), in which $a = -0.218\Delta S^\circ$, a plot of pK_a' (25 °C) against pK_a' (85 °C) should be linear with slope 1.25. This is

$$pK_a'(85°C) = 1.25\,pK_a'(25°C) - 0.25a \qquad (12)$$

found to be so,[69] and the derived value of a (5.4) converts to $\Delta S^\circ = -25$ cal $K^{-1}mol^{-1}$, much more negative than is the case for univalent cations, and consistent with the fact that experimental values of ΔS° lie in the range $-(13—22)$ cal $K^{-1}mol^{-1}$.[56] Although this equilibrium, unlike the first, is not isoelectric, the differences in the solvation requirements of BH_2^+ and B^- are probably approximately constant. Equation (13) therefore describes the temperature dependence of the acidity constant K_a'.

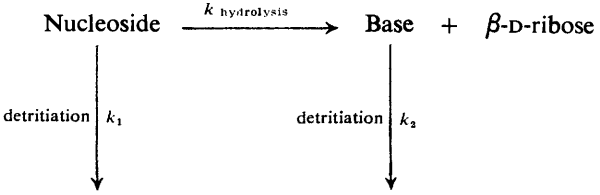
$$-\frac{d(pK_a')}{dT} = \frac{pK_a' - 5.4}{T} \qquad (13)$$

10 Miscellaneous

Although the C-8 hydrogen in purines is not sterically hindered in any way, the same is not true in polynucleotides, such as ribonucleic acids; here each nucleotide base is in a different environment, governed by the primary structure and secondary factors. The ways in which these considerations can influence rates of exchange have been studied by both Maslova[70] and Schimmel[71] and their respective co-workers, and it is clear that not only are the rates extremely sensitive to the local microenvironment but that the method offers a powerful way of studying protein–nucleic acid interactions, and should be applicable to a wide variety of systems.

[68] (*a*) D. D. Perrin, *Aust. J. Chem.*, 1964, **17**, 484; (*b*) D. D. Perrin, *J. Chem. Soc.*, 1965, 5590.
[69] J. R. Jones and S. E. Taylor, *J. Chem. Res.*, 1980, (S) 154.
[70] (*a*) R. N. Maslova, E. A. Lesnik, and Ya. M. Varshavsky, *Biochem. Biophys. Res. Commun.*, 1969, **34**, 260; (*b*) *Mol. Biol.*, 1969, **3**, 575; (*c*) *FEBS Lett.*, 1969, **3**, 211; (*d*) E. A. Lesnik, R. N. Maslova, T. G. Samsonidze, and Ya. M. Varshavsky, *FEBS Lett.*, 1973, **33**, 7; (*e*) G. N. Lapiashvili, E. A. Lesnik, R. N. Maslova, and Ya. M. Varshavsky, *Nucleic Acids Res.*, 1977; **4**, 2181.
[71] (*a*) R. C. Gamble and P. R. Schimmel, *Proc. Natl. Acad. Sci. USA*, 1974, **71**, 1356; (*b*) R. C. Gamble, H. J. P. Schoemaker, E. Jekowsky, and P. R. Schimmel, *Biochemistry*, 1976, **15**, 2791; (*c*) H. J. P. Schoemaker, R. C. Gamble, G. P. Budzik, and P. R. Schimmel, *Biochemistry*, 1976, **15**, 2800; (*d*) P. R. Schimmel, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 1979, **50**, 187.

Rates of detritiation from the C-8 position of purines, as for other exchange reactions, invariably give first-order kinetics. Departure from this behaviour usually signifies the onset of another reaction, and in the case of various purine nucleosides the curvature of the first-order plots can be used to obtain the acid-catalysed hydrolysis rate constant, when analysed according to the sequence shown in Scheme 2.[72]



Scheme 2

A novel method of assaying the guanine content of DNA has been devised[73] that takes advantage of the extreme lability of the C-8 proton in 7-methylguanosine. The procedure entailed biosynthetically labelling DNA using [8-^{3}H]guanosine, followed by methylation with dimethylsulphate; the tritium was rapidly released from the 7-methyl-[8-^{3}H]guanosine residues.

11 Concluding Remarks

The biochemists' frequent preference for ^{14}C- rather than ^{3}H-labelled compounds, despite the fact that their preparation is usually more demanding in time, skill, and expense, is more often than not based on the dangers associated with possible adventitious hydrogen exchange reactions. The chemist, on the other hand, finds that these reactions can be a useful way of investigating details of reaction mechanisms. During such studies one can gain an appreciation of the many factors that can influence the exchange rates, and how these can be applied to good effect. Consequently, when such studies are carried out on important and interesting compounds, such as the purines, one is presented with a fertile area of research, both pure and applied.

Various aspects of the work described here have been supported by Amersham International (formerly The Radiochemical Centre), the Science Research Council, and NATO.

[72] J. R. Jones and S. E. Taylor, *Int. J. Chem. Kinet.*, 1980, **12**, 141.
[73] M. Tomasz, *Biochim. Biophys. Acta*, 1970, **199**, 18.