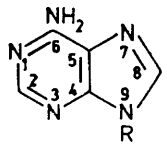
Proton Transfer from Heterocyclic Compounds. Part III.[1] Adenine and Adenosine

By **John A. Elvidge, John R. Jones,*** and **Conor O'Brien,** Chemistry Department, University of Surrey, Guildford
E. Anthony Evans and **Hilary C. Sheppard,** The Radiochemical Centre, Amersham

Rates of detritiation from C-8 of adenine and adenosine have been measured over a pH range at 85°. Analysis of the data enables the pK_a values for protonation at N-7 to be calculated. For adenine, measurements have been made at several temperatures and the observed entropy of activation, taken in conjunction with other available information, supports a mechanism involving rate-determining hydroxide ion attack on the N-7 protonated species. For adenosine at high pH, an additional mechanism involving hydroxide catalysed exchange of the neutral compound is important.

ADENINE (1a), a highly resonance stabilised purine, contributes to the structures of the nucleic acids and is a constituent of widespread nucleotides and transfer coenzymes. Labelled adenine is consequently of importance for biochemical and mechanistic studies, but for the successful use of such tracers, knowledge of the stability



(1)
a; R=H
b; R=β-D-ribofuranosyl

of the label is essential to prevent erroneous conclusions being drawn.[2] The 8-H of purines can undergo isotopic exchange,[3] providing, for example, a ready means of labelling adenine. In order to define the conditions under which 8-H labelled adenine may be employed we have studied the rates of detritiation from C-8 of adenine. A similar study has been undertaken for adenosine (1b) because a comparison of the results with those for adenine makes possible a correct appraisal of the importance of the β-D-ribofuranosyl group in respect of isotopic exchange from C-8.

Since Eidinoff's [4] initial observation that tritium could be incorporated into adenine merely by heating a solution of the compound in tritiated water in the presence of a platinum catalyst at 100° for 18 h, several reports of isotopic exchange in both this compound and adenosine have been reported. Thus exchange at C-8 of 7- and 9-benzyladenines and adenosine was achieved by refluxing in deuterium oxide or a D_2O–dimethylformamide mixture;[5,6] prolonged heating at 100° in the case of 3-benzyladenine [6] led to exchange at C-2 as well as at C-8. Subsequent studies [2,7] have shown that exchange from C-2 of adenine is always slower than from C-8.

In the case of adenosine (1b), the available results are somewhat conflicting. Thus Wechter,[8] in an attempt to develop methods for preparing specifically labelled nucleosides and nucleotides, found that at 95° the rate of deuteriation at C-8 of adenosine increased with pH until it became immeasurably fast in 0·1N-potassium hydroxide whereas Van Dyke [9] was unable to detect any loss of tritium from [8-^{3}H]adenosine in 1N-potassium hydroxide over a period of 20 h at 37°. This difference is probably related to the fact that adenosine hydrolyses in 0·1N-potassium hydroxide over the course of 1 h at 100° whereas adenine is stable in 1N-alkali at 100° for a much longer period.[10] Tomasz and co-workers [11] found that the rates of detritiation of [8-^{3}H]adenosine were virtually constant over the pH range 4—11; Shelton and Clark [12] observed that the rates were approximately constant at pH 2, 7·5, and 11 whereas Kawazoe [13] found that the rate of deuteriation decreased below pD 4 but increased abruptly at between pD 12 and 13. Other studies [14] report exchange of adenosine monophosphate at a single pH.

EXPERIMENTAL

Materials.—[8-^{3}H]adenine (500 mCi mmol^{-1}; solid) and generally labelled [^{3}H]adenosine (843 mCi mmol^{-1}; aqueous solution) were from the Radiochemical Centre, Amersham.

Kinetics.—Details of the methods used to follow the rates of detritiation have been given.[1,15] When the reaction half-lives were greater than 6 h an initial rate method was employed and the pseudo-first-order rate constant k_{obs} obtained from the slope ($k_{obs}c_\infty$) of the plot of radioactivity of tritiated water (dis. min^{-1}) against time. For faster reactions, the increase in the radioactivity of the water was followed over >75% of the reaction and k_{obs} determined from the slope ($-2{\cdot}303\,k_{obs}$) of the plot of $\log_{10}(c_\infty - c_t)$ against time. The only difference from previous work was that the adenosine

[1] Part II, J. A. Elvidge, J. R. Jones, C. O'Brien, E. A. Evans, and H. C. Sheppard, *J.C.S. Perkin II*, 1973, 1889.
[2] E. A. Evans, H. C. Sheppard, and J. C. Turner, *J. Labelled Compounds*, 1970, **6**, 76.
[3] M. P. Schweizer, S. I. Chan, G. K. Helmkamp, and P. O. P. Ts'o, *J. Amer. Chem. Soc.*, 1964, **86**, 696.
[4] M. L. Eidinoff and J. E. Knoll, *J. Amer. Chem. Soc.*, 1953, **75**, 1992.
[5] F. J. Bullock and O. Jardetsky, *J. Org. Chem.*, 1964, **29**, 1988.
[6] J. R. Fox, Ph.D. Thesis, Illinois, 1965.
[7] J. A. Elvidge, J. R. Jones, C. O'Brien, and E. A. Evans, *Chem. Comm.*, 1971, 394.
[8] W. J. Wechter, *Coll. Czech. Chem. Comm.*, 1970, **35**, 2003.
[9] K. Van Dyke, C. Szustkiewicz, C. H. Lantz, and L. H. Saxe, *Biochem. Pharmacol.*, 1969, **18**, 1417.
[10] A. S. Jones, A. M. Mian, and R. T. Walker, *J. Chem. Soc.* (*C*), 1968, 692.
[11] M. Tomasz, J. Olson, and C. M. Mercado, *Biochemistry*, 1972, **11**, 1235.
[12] K. R. Shelton and J. M. Clark, *Biochemistry*, 1967, **6**, 2735.
[13] M. Maeda, M. Saneyoshi, and Y. Kawazoe, *Chem. Pharm. Bull.*, 1971, **19**, 1641.
[14] R. N. Maslova, E. A. Lesnick, and Ya. M. Varshavsky, *Biochem. Biophys. Res. Comm.*, 1969, **34**, 260; C. C. McDonald and W. D. Philips, *Biopolymers*, 1965, **3**, 609.
[15] J. A. Elvidge, J. R. Jones, C. O'Brien, E. A. Evans, and J. C. Turner, *J.C.S. Perkin II*, 1973, 432.

used contained tritium at C-2 as well as at C-8. We had previously found [7] that tritium at C-2 exchanged *ca.* 2000 times slower in H_2O at 85° than from C-8. It was therefore assumed that exchange from this position was complete before any tritium was lost from C-2.

The percentage tritium at C-8 was determined by first assaying the tritium content of a dilute solution of the generally labelled adenosine in water and then comparing it with the tritium content of the tritiated water of a similar solution which had been kept at 85° for 24 h. At the low adenosine concentrations employed, quenching was unimportant and the ratio of the tritium content of the water to that of the adenosine gave the fraction of tritium at C-8. An average of five determinations gave 81 ± 3%.

RESULTS AND DISCUSSION

The bell-shaped rate–pH profile observed for adenine exchange (Table 1 and Figure 1) is reminiscent of that

TABLE 1

Rate–pH data for [8-^{3}H]adenine (1a), and [8-^{3}H]adenosine (1b) at 85°

pH (at 85°)	(1a) $10^6k_{obs}/s^{-1}$	(1b) $10^6k_{obs}/s^{-1}$
2·05	2·26	7·40
2·55	4·50	19·1
3·00		34·5
3·12	11·8	
3·53		57·0
3·60	18·5	
3·70	20·4	
3·90	24·1	
4·10	26·4	63·0
4·65	32·4	
6·25	33·0	66·6
8·38	29·2	
9·05	20·4	
9·17	9·85	
9·30	8·10	
9·42	4·00	
9·50		86·3
10·10	3·06	
10·39	2·02	
10·49		277
10·80		435
10·98		625
11·19		986
11·50		1440

previously obtained for the structurally similar benzimidazole [15] and purine.[1] In both cases rate-determining attack by hydroxide ion on the protonated substrate occurs so that if the same mechanism applies here we have equation (1) which leads to (2) where $K_a =$ $[BH][H^+]/[BH_2^+]$ and $K_a' = [B^-][H^+]/[BH]$ with BH_2^+, BH, and B^- representing protonated adenine, the neutral molecule, and the monoanion, respectively; k_{obs} is the pseudo-first-order rate constant for the detritiation of [8-^{3}H]adenine.

$$\text{Rate} = k[BH^+][OH^-] \quad (1)$$

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

Equation (2) simplifies to (3) when $K_a' \ll [H^+]$ and

$$k_{obs} = kK_W/(K_a + [H^+]) \quad (3)$$

$K_aK_a' \ll [H^+]^2$. The relative rate (R), which is defined as a fraction of the rate in the pH-independent region (where $k_{obs} = kK_W/K_a$) is then given by equation (4).

$$R = K_a/(K_a + [H^+]) \quad (4)$$

Rearranging and taking logarithms leads to equation (5)

$$pH = pK_a + \log_{10}[R/(1 - R)] \quad (5)$$

so that a plot of pH against $\log_{10}[R/(1 - R)]$ should give

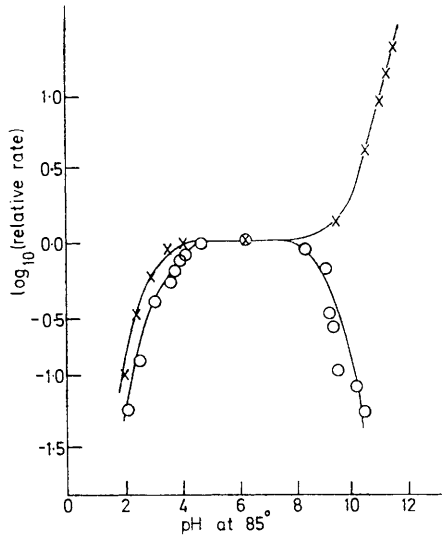


FIGURE 1 Rate–pH profile for [8-^{3}H]adenine (○), and [8-^{3}H]-adenosine (×) at 85°. The calculated line is drawn using values given in the text

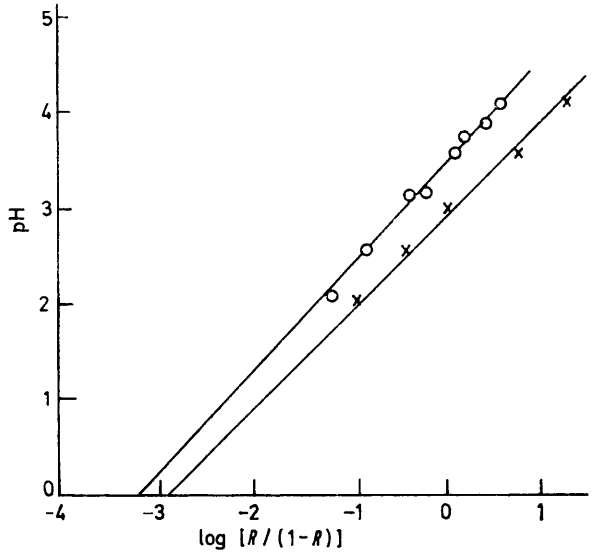


FIGURE 2 Plot of pH against $\log[R/(1 - R)]$ for [8-^{3}H]-adenine (○) and [8-^{3}H]adenosine (×)

a straight line of unit slope and an intercept at pH = 0 of $-pK_a$. The results for adenine (Figure 2) do in fact give such a slope and a pK_a of 3·2. This latter finding may be compared with the result obtained from the calculated curve (Figure 1) which was constructed using

equation (6). The best fit with the experimental data

$$R = \frac{K_a}{K_a + \frac{K_a K_a'}{[H^+]} + [H^+]} \quad (6)$$

was obtained using $pK_a = 3{\cdot}5$ and $pK_a' = 9{\cdot}0$. The corresponding values at 25° are 4·20 and 9·87 [16] respectively reducing to $3{\cdot}5_4$ and 8·1 at 85° if the semi-empirical Perrin equation [17] is employed. The behaviour of adenosine (Table 1 and Figure 1) is similar to that of adenine up to pH *ca.* 8 but at higher values the rates of detritiation instead of falling off increase dramatically. This difference can be ascribed to the fact that adenosine like the 9-alkylpurines does not have an ionisable 9-NH group *i.e.* $K_a' = 0$. The rate increase, which incidentally was not observed by Tomasz,[11] is due to the onset of a second reaction pathway involving the hydroxide ion and the neutral substrate. We then have equation (7)

$$\text{Rate} = k[BH^+][OH^-] + k'[B][OH^-] \quad (7)$$

leading to (8). Equation (8) which was used to construct

$$k_{obs} = \frac{kK_W}{K_a + [H^+]} + \frac{k'K_a[OH^-]}{K_a + [H^+]} \quad (8)$$

the calculated curve reduces to (9) when $[H^+] \ll K_a$;

$$k_{obs} = kK_W/K_a + k'[OH^-] \quad (9)$$

$k'(1{\cdot}92 \times 10^{-2}$ l mol^{-1} s^{-1}) was obtained from the plot of k_{obs} against $[OH^-]$, and the pK_a value that gives the best fit with the experimental results was found to be 2·9 by a trial-and-error procedure. The value is the same as that obtained from a plot of pH against $\log_{10}[R/(1-R)]$ (Figure 2). The literature value [16] at 25° (3·50) reduces to 3·0 at 85° if the Perrin equation [17] is employed. The other pK_a value for adenosine (12·35 at 25°) refers to ionisation in the β-D-ribofuranosyl group but, as the results in Figure 1 show, there is no need to make any allowance for this factor over the pH range studied.

As with purine the only uncertainty with regard to the reaction mechanism concerns the site of protonation. *X*-Ray crystallographic investigations of adenine hydrochloride,[18] adenosine-5′-phosphate,[19] adenosine-3′-phosphate,[20] and adenosine-2′-uridine-5′-phosphate [21] show that in the crystalline state they are all protonated at N-1. However, recent work by Chan and Nelson [22] on the effect of pH on the ^{1}H n.m.r. parameters of adenyl-(3′,5′)-adenosine suggests that several monoprotonated species exist in equilibrium with one another.* It is also significant that when adenine is alkylated in neutral solution with ethyl methanesulphonate 3-, 9-, and 1-ethyl isomers are produced in yields of 25, 9, and 8% respectively.[23] Similarly when adenosine is alkylated with dimethyl sulphate the 7-methyl- as well as the predominant 1-methyl-adenosine is produced.[24] As for purine we favour a mechanism in which the reactive species is the N-7 protonated form, which is assumed to be present to a small extent.

The rates of detritiation from C-8 of adenine have been measured over a 35° temperature range (Table 2), and the

TABLE 2

Rates of detritiation of [8-^{3}H]adenine in H_2O at various temperatures

t/°C	$10^6 k_{obs}$/ s^{-1}	$10^4 K_a$	$10^{14} K_W$	$10^{-3} k_2$/ l mol^{-1} s^{-1}
50	1·13	1·3	5·5	2·46
65	4·87	$1{\cdot}7_5$	12·6	6·73
75	13·9	$2{\cdot}2_5$	20·0	15·6
85	33·0	2·9	31·6	30·2

observed activation energy [7] found to be 22·3 kcal mol^{-1} from a plot of $\log_{10} k_{obs}$ against T^{-1}. However it is more informative if the second-order rate constant k ($= k_{obs}K_a/K_W$) for reaction between protonated substrate and hydroxide ion is plotted against T^{-1}. In this case the activation energy is $16{\cdot}5 \pm 0{\cdot}8$ kcal mol^{-1} and $\Delta S^{\ddagger} + 7 \pm 1$ cal K^{-1} mol^{-1}. Although the magnitude of the activation energy is of interest, considerably more importance attaches to the entropy of activation. This is because of the role of substrates with the purine functions in enzyme reactions, and the fact that base stacking effects [14] make the solvent less accessible to the substrate in more concentrated solutions. In three other reactions (which probably occur by the same mechanism) involving guanine, thiazole, and 1-ethyltetrazole (Table 3) all the values of $\Delta S^{\ddagger}$ are large and positive. This is in sharp contrast to the values obtained from reactions in which the water acts both as the solvent and the base, and where the carbon acid carries no net charge; in fact, in terms of charge distribution, one is the reverse of the other, with the reactions between uncharged carbon acids and the hydroxide (or alkoxide) ion occupying an intermediate position.

The entropies of activation, which cover more than 60 cal K^{-1} mol^{-1} as the charge type varies, can be interpreted in terms of differences in solvation of the reactants and transition state. Thus in the detritiation of adenine, the protonated molecule and especially the hydroxide ion will be solvated but the transition state will be solvated to a much smaller extent because it has zero net charge. The process of desolvation is therefore accompanied by an increase in entropy and the magnitude of $\Delta S^{\ddagger}$ will depend not only on substrate reactivity (the faster the reaction the earlier will the transition state occur on the reaction co-ordinate and the less advanced the desolvation) but also on the importance of non-equilibrium

* Unfortunately serious doubts now exist concerning the validity of some of this work (M. Pieber, P. A. Kroon, J. H. Prestegard, and S. I. Chan, *J. Amer. Chem. Soc.*, 1973, **95**, 3408).

[16] J. J. Christensen, J. H. Rytting, and R. M. Izatt, *Biochemistry*, 1970, **9**, 4907.
[17] D. D. Perrin, *Austral. J. Chem.*, 1964, **17**, 484.
[18] W. Cochran, *Acta Cryst.*, 1951, **4**, 81.
[19] J. Kraut and L. H. Jensen, *Acta Cryst.*, 1963, **16**, 79.
[20] M. Sundaralingam, *Acta Cryst.*, 1966, **21**, 495.
[21] E. Shefter, M. Barlow, R. Sparks, and K. Trueblood, *J. Amer. Chem. Soc.*, 1964, **86**, 1872.
[22] S. I. Chan and J. H. Nelson, *J. Amer. Chem. Soc.*, 1969, **91**, 168.
[23] B. C. Pal, *Biochemistry*, 1962, **1**, 558.
[24] P. D. Lawley and P. Brookes, *Biochem. J.*, 1964, **92**, 19C.

transition state solvation.[25] It has previously been suggested [42] that $\Delta S^{\ddagger}$ for a number of rate-determining proton transfers in water is more negative than expected and that this may be because desolvation of the proton does not take place before the transition state is reached. The data in Table 3 clearly show that the entropies of activation for hydroxide catalysed deprotonations in water are consistently more negative than for methoxide catalysed deprotonations in methanol but the near zero values in the latter case may owe something to the importance of the internal return mechanism.[34] Values of $\Delta S^{\ddagger}$ for the ionisation of several nitroaromatic compounds in ethanol–ethoxide solutions are also close to zero. Proton transfer between two neutral substrates on the other hand will result in an increase in solvation as the transition state resembles an ion pair; the process of solvation will make a negative contribution to $\Delta S^{\ddagger}$.

There are several exceptions to the apparent trends observed in Table 3, most notably the hydroxide catalysed detritiation of phenylacetylene [43] where $\Delta S^{\ddagger}$ is +42 cal K^{-1} mol^{-1} rather than the somewhat negative values characteristic of many reactions of this kind. The entropy criterion should of course be exercised with caution and preferably in conjunction with other criteria. Finally $\Delta S^{\ddagger}$ for the reaction between protonated adenine and hydroxide ion is a composite function which includes contributions from the protonation equilibrium [16] ($\Delta S^{0} = +3{\cdot}2$ cal K^{-1} mol^{-1} at 25°) and the ionisation of water [44] (ΔS^{0} at 85° = −27 cal K^{-1} mol^{-1}).

TABLE 3

Entropies of activation for various proton transfer reactions from carbon acids (BH)

Reaction mechanism	Carbon acid	$\Delta S^{\ddagger}$/cal K^{-1} mol^{-1}	Ref.
$BH + H_2O \longrightarrow B^- + H_3O^+$	Ethyl 2-oxocyclopentanecarboxylate	−38	25
	Malononitrile	−22	26
	t-Butylmalononitrile	−21	26
	Benzoylacetone	−33	27
$BH + OH^- \longrightarrow B^- + H_2O$	Nitroethane	−15	28
	1,4-Dicyano-but-2-ene	−12	29
	Acetone	−21	30
	Acetophenone	−19	31
$BH + OMe^- \longrightarrow B^- + MeOH$	Fluorene	−1	32
	7*H*-Benzo[*c*]fluorene	−2	32
	Diphenylmethane	0	33
	Triphenylmethane	0	33
	1,3-Difluorobenzene	2, −4	34, 35
	1*H*-Undecafluorobicyclo[2.2.1]heptane	−4	36
$BH + OEt^- \longrightarrow B^- + EtOH$	4-Nitrobenzyl cyanide	−1	37
	1,3,5-Trinitrobenzene	0	38
$BH_2^+ + OH^- \longrightarrow BH + H_2O$	Adenine	+7	Present work
	1-Ethyltetrazole	+22	39
	Guanine	+10	40
	Thiazole	*ca.* 40 [a]	41

[a] Subject to large uncertainty.

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

[3/1336 *Received*, 25*th June*, 1973]

[25] J. R. Jones, *Trans. Faraday Soc.*, 1969, **65**, 2430.
[26] F. Hibbert, F. A. Long, and E. A. Walters, *J. Amer. Chem. Soc.*, 1971, **93**, 2831.
[27] J. R. Jones and S. P. Patel, to be published.
[28] R. G. Pearson, *J. Amer. Chem. Soc.*, 1948, **70**, 204.
[29] E. A. Walters and F. A. Long, *J. Amer. Chem. Soc.*, 1969, **91**, 3733.
[30] J. R. Jones, *Trans. Faraday Soc.*, 1965, **61**, 95.
[31] J. R. Jones, R. E. Marks, and S. C. Subba Rao, *Trans. Faraday Soc.*, 1967, **63**, 111.
[32] A. Streitwieser, jun., W. B. Hollyhead, A. H. Pudjaatmaka, P. H. Owens, T. L. Kruger, P. A. Rubenstein, R. A. MacQuarrie, M. L. Brokaw, W. K. C. Chu, and H. M. Niemeyer, *J. Amer. Chem. Soc.*, 1971, **93**, 5088.
[33] A. Streitwieser, jun., W. B. Hollyhead, G. Sonnichsen, A. H. Pudjaatmaka, C. H. Chang, and T. L. Kruger, *J. Amer. Chem. Soc.*, 1971, **93**, 5096.
[34] A. Streitwieser, jun., J. A. Hudson, and F. Mares, *J. Amer. Chem. Soc.*, 1968, **90**, 648.
[35] J. Hine and P. B. Langford, *J. Org. Chem.*, 1962, **27**, 4149.
[36] A. Streitwieser, jun., and D. Holtz, *J. Amer. Chem. Soc.*, 1967, **89**, 692.
[37] E. F. Caldin, M. Kasparian, and G. Tomalin, *Trans. Faraday Soc.*, 1968, **64**, 2802.
[38] F. F. Caldin and G. Long, *Proc. Roy. Soc.*, 1955, **228A**, 263.
[39] A. C. Rochat and R. A. Olofson, *Tetrahedron Letters*, 1969, 3377.
[40] C. O'Brien, Ph.D. Thesis, Surrey, 1972.
[41] R. A. Coburn, J. M. Landesberg, D. S. Kemp, and R. A. Olofson, *Tetrahedron*, 1970, **26**, 685.
[42] M. M. Kreevoy and R. A. Kretchmer, *J. Amer. Chem. Soc.*, 1964, **86**, 2435.
[43] C. Eaborn, G. A. Skinner, and D. R. M. Walton, *J. Chem. Soc.* (*B*), 1966, 922.
[44] T. Ackermann, *Z. Electrochem.*, 1958, **62**, 411.