Proton Transfer from Heterocyclic Compounds. Part 11.[1] Histidine, Histamine, *N*-Acetylhistidine, and Histidine-containing Peptides

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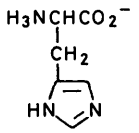
Rates of detritiation from the C-2 position of [2-^{3}H]histidine, histamine, and *N*-acetylhistidine as well as a number of histidine-containing peptides (glycyl-L-histidine, glycyl-L-histidylglycine, β-alanylhistidine, histidylserine, and histidyltyrosine) have been measured over a pH range at 85°. For both histidine and histamine the rate–pH profiles are indicative of hydroxide ion attack on both the protonated and ' neutral ' species. The magnitude of the rate constant for the second mechanism indicates that the kinetically equivalent zwitterionic form of the substrate also participates in the reaction. The results for *N*-acetylhistidine can be represented by a bell-shaped rate–pH profile, consistent with hydroxide ion attack on the N-3 protonated species. The results for the other peptides can, to a first approximation, be represented by the same profile, but this may be an over-simplification, resulting from closely similar pK_a values. Electrostatic factors arising from the presence of charged carboxylate and amino-groups close to the C-2 exchanging position are negligible.

HISTIDINE plays an important part in determining the mode of action of numerous proteins.[2] In order to understand fully the way in which they work a knowledge of the environment and pK_a values of the histidine groups is necessary. Unlike most other amino-acids where ionisation and protonation is usually restricted to the carboxy- and amino-groups respectively histidine contains the imidazole function which can serve both as an acid and a base in the physiological pH range. In view of the fact that the 2-H can undergo isotopic exchange one is therefore provided with an excellent opportunity of studying the factors that can influence the exchange rates. The present study is concerned with measuring rates of detritiation from the C-2 position of histidine (1), histamine (2), *N*-acetylhistidine (3), and a number of histidine-containing peptides [glycyl-L-histidine (4), glycyl-L-histidylglycine (5), β-alanylhistidine (6), histidylserine (7), and histidyltyrosine (8)].

In contrast to imidazole, where several isotopic exchange studies [3-7] have been reported, few investigations have been carried out on histidine and histidine-containing compounds. Bradbury *et al.*[8] published preliminary details which indicated that differences in rates could be ascribed to electrostatic factors resulting from the presence of charged groups close to the exchanging position and Markley and Cheung [9] showed that proton abstraction is involved in the rate-determining step during tritium exchange into the tripeptide glycyl-L-histidylglycine.

More qualitative observations of isotopic exchange stem from the realisation [10] that of all the hydrogen atoms in a native protein the most easily resolvable are the nitrogen-bound protons of the tryptophan indole group and the C-2 protons of the histidine imidazole group. N.m.r. spectroscopy therefore provides a means not only of measuring the pK_a values of histidine residues in proteins [9-11] but of using differential rates of exchange [12,13] to assign the positions of the different histidines; cleavage and separation of the peptides must however be carried out under mild conditions where back exchange is minimal. By adopting this approach Krieger *et al.*[14] have shown that the half-times for tritium exchange into the histidine residues of trypsin are between 20 and 300 times slower than for histidine itself.



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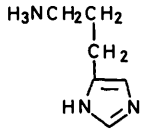

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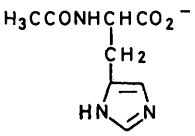

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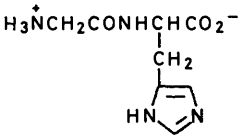

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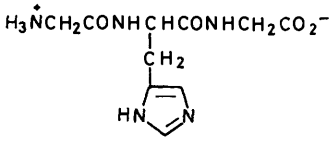

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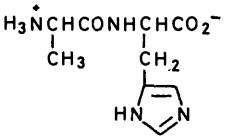

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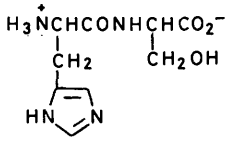
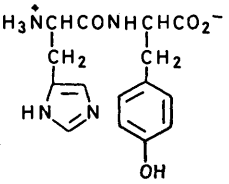




EXPERIMENTAL

Materials.—All the compounds were commercially available and their purity was checked prior to use. In general tritiation was effected by keeping between 50 and 100 mg of the substrate (in the case of glycyl-L-histidine as the hydrochloride) and tritiated water (5—10 μl, 50 Ci ml^{-1}) in a sealed tube at 85° for 3—14 h. In cases such as histidine,

where the solubility was low a small amount of dimethyl sulphoxide was added. The solvent was then lyophilised and the product washed (at least twice) with water to remove very labile tritium before the solvent was lyophilised for the final time.

Kinetics.—The rates of detritiation were measured over the reported pH range at 85° using the previously described procedures.[15] Monitoring of the u.v. spectra of the reaction solutions over the duration of the detritiation experiments served to confirm that no side reactions occurred.

RESULTS AND DISCUSSION

The rate–pH profile for isotopic hydrogen exchange from the C-2 position of imidazole is bell-shaped,[4-6] with an intermediate pH range where the rate is virtually unaffected by changes in pH. A similar profile has been observed for benzimidazole.[14] Consequently the sigmoidal curve obtained for histidine (Figure 1) must

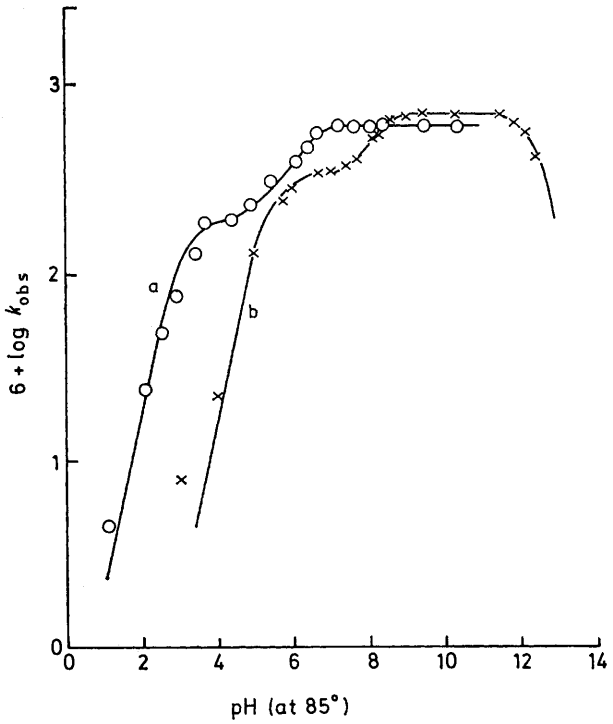


FIGURE 1 Rate–pH profiles for the detriation of (a) [2-^{3}H]histidine, (b) [2-^{3}H]histamine in aqueous buffers at 85°. The drawn curves are computed from equation (7) with values given in the text. The histidine results have been displaced by −2 pH units

imply the involvement of one or other of the ionisable groups in the side-chain. Furthermore the fact that histamine gives a closely similar rate–pH profile suggests that it is the amino-group that is responsible. The experimental results (Table 1) can therefore be analysed in the following manner. The pK_a for ionisation of the carboxylic group in histidine [16] has a value of 2.2 at 21°. Consequently in the pH range under study histidine can exist in four forms: (1) the ' neutral ' form ($NH_2ImHCO_2^-$) or BH, (2) the amino-protonated form ($\overset{+}{N}H_3ImHCO_2^-$) or BH_2^+, (3) the doubly protonated, both on the N-3 of the imidazole group and the amino-

TABLE 1

Detritiation rate constants ($k_{obs.}$) for [2-^{3}H]histidine (1) and [2-^{3}H]histamine (2) in aqueous buffers at 85°

(1)		(2)	
pH (at 85°)	$10^5k_{obs.}/s^{-1}$	pH (at 85°)	$10^5k_{obs.}/s^{-1}$
3.06	0.45	3.01	0.84
4.03	2.5	4.02	2.27
4.46	4.9	4.96	12.9
4.81	7.8	5.70	23.9
5.34	13.2	5.95	28.3
5.61	19.2	6.64	32.9
6.34	19.4	7.00	33.8
6.80	23.9	7.39	36.5
7.34	31.4	7.73	39.5
8.04	41.1	8.09	51.2
8.32	48	8.27	53
8.55	56	8.59	64.9
9.13	60.9	9.00	67.2
9.39	60.1	9.40	68.7
9.97	60.9	10.33	67.2
10.37	62.4	11.47	67.2
11.39	62.0	11.83	62.4
12.31	60.5	12.18	55.3
		12.45	40.4

TABLE 2

Detritiation rate constants ($k_{obs.}$) for [2-^{3}H]-*N*-acetylhistidine (3) and [2-^{3}H]histidine-containing peptides in aqueous buffers at 85°

Compound	(4)	(5)	(3)	(6)	(7)	(8)
pH (at 85°)	←		10^5 $k_{obs.}/s^{-1}$			→
2.89			0.18			
3.01	1.8					
3.07		0.26				
4.00		1.73	1.07	4.1		
4.10					2.46	2.26
4.50	2.64					
4.64		6.87				
5.02		13.0				
5.20	18.4		15.0			12.6
5.25				16.7		
5.32		28.5	18.6			
5.58			34.5	35.2		
5.64	26.6					
5.70		39.5		46.9		
5.82			53.0			
6.07	58.8					
6.25		52.0	64.0	61.4	39.0	33.3
6.34				63.5		
6.70	74.4					
7.20			79.7			
7.36				73.4		
7.53		54.1				
7.71				74.8		
8.07			84.5			
8.43			88.1	78.6		
8.61	81.8					
8.65		55.8				
8.70					75.4	74.7
8.95	81.0					
9.08			91.7			
9.44			89.2	78.3		
10.02		55.6				
10.05			91			
10.19			90			
10.44	82			80		
10.58			87			
11.47	80	55		80		
11.54			79			
11.74					61.2	76
11.96				69		
12.09			65.4			
12.19	69	47				
12.37			56.0	54		
12.50	49.3	36.7				

group of the side chain, form ($NH_3^+ImH_2^+CO_2^-$) or BH_3^{2+}, and (4) the anionic form, as a result of the ionisation of the N-1 hydrogen ($NH_2Im^-CO_2^-$) or B^-. The concentrations of these different forms are related by means of

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

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

the acidity constants K_a, K_a', and K_a'' where $K_a = [BH_2^+][H^+]/[BH_3^{2+}]$, $K_a' = [BH][H^+]/[BH_2^+]$, and $K_a'' = [B^-][H^+]/[BH]$.

The total histidine concentration is given by equation (1) which leads to (2). Consequently the BH_3^{2+} concentration is given by equation (3) and in a similar manner equation (4) is also obtained. Assuming a rate

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

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

$$\text{Rate} = k[BH_3^{2+}][OH^-] + k'[BH_2^+][OH^-] \quad (5)$$

equation of the form (5) we obtain equation (6). Hence equation (7) obtains. For $K_a \gg [H^+] \gg K_a', K_a''$

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

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

and $k \gg k'$, $k_{obs.} = kK_w/K_a$ corresponding to the first plateau on the rate–pH profile (pH 5.5—6.5). Similarly for $[H^+] \ll K_a'$ and $k \gg k'$, $k_{obs.} = k'K_w/K_a'$, corresponding to the second plateau at pH 9—12. The calculated lines in Figure 1 are constructed using equation (7) and a trial and error procedure. The pK_a values that give the best fit are given in Table 3 and compare well with the literature values [16-18] once the necessary temperature adjustment has been made using the Perrin equation.[19]

The derived rate constants (k) for histidine and histamine (Table 3) are less than a factor of two different, so that the inhibiting effect of a negatively charged carboxylate group is small. Similarly the positively charged amino-group would be expected to have very little influence and this is confirmed by the fact that the rate constant for the doubly protonated species is consistent with that obtained for mono-protonated species having similar pK_a' values, *e.g.* in the case of benzimidazole,[15] pK_a' = 4.60 and $k = 6.5 \times 10^4$ l mol^{-1} s^{-1}.

TABLE 3

Derived acidity and rate constants (l mol^{-1} s^{-1}) at 85° for histidine, histamine, *N*-acetylhistidine, and histidine-containing peptides

	$10^{-3}k$	k'	pK_a	pK_a' (kinetics)	pK_a''	pK_a (lit. values) *	pK_a' (lit. values) *	pK_a'' (lit. values) *	Ref.
Histidine	6.1	49	5.0	7.6		6.17	9.28		16
Histamine	10	24	4.9_5	7.9_5	12.8	6.04 † (5.01)	9.75 † (7.97)	14.35 †	17
Glycyl-L-histidine	6.5		5.6		12.8	6.85 (5.56)	8.33		16
Glycyl-L-histidylglycine	9.8		5.2_5		12.8	6.63 (5.38)	8.17		16
N-Acetylhistidine	3.5		5.9		12.6				
β-Alanylhistidine	3.5		5.8_5		12.7	6.58 ‡ (5.76)	9.04 ‡		18

* Refers to 25° unless otherwise stated. † 21°, pK values in parentheses refer to 85° having been obtained using the literature values in conjunction with the Perrin equation.[19] ‡ 37°.

The main interest in the histidine and histamine results lies in the magnitude of the rate constants (k') for the reaction between the hydroxide ion and the amino-protonated species. If we can assume that the electrostatic effect arising from protonation of the amino-group is small then the substrate can be treated effectively as a neutral entity. In previous studies in this series we have shown that compounds which react in the neutral form and which can not form zwitterions have k' values in the range 10^{-1}—10^{-3} l mol^{-1} s^{-1}, *e.g.* 9-isopropylpurine [20] (1.5×10^{-2}), adenosine [21] and 1-methylinosine [22] (1.9×10^{-2}), and caffeine [23] (2.7×10^{-2}). The ratio k/k' for these compounds is in the region 10^7—10^8. Consequently the high values of k' for both histidine and histamine (and resulting low k/k' ratio) once again point to the involvement of a kinetically equivalent species such as a zwitterion, *via* the equilibrium (8). Resonance (9) in the imidazolium cation will probably stabilise the second zwitterion.

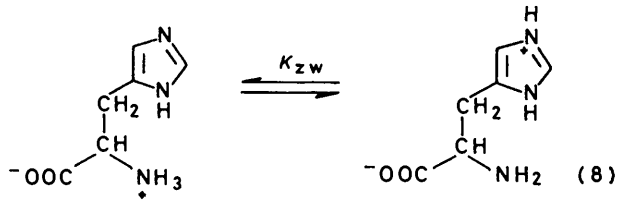
For both histidine and histamine the rate constant k' is therefore a composite function [equation (10)] where k_0 and $k_\pm$ are the rate constants for hydroxide ion attack on the 'neutral' and zwitterionic forms, respectively.

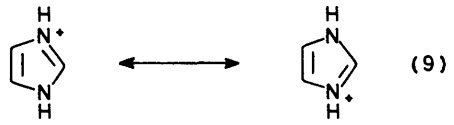
The possible involvement of the latter in hydrogen isotope exchange reactions involving heterocyclic carbon acids was first suggested by Tomasz [24] and recent studies on the xanthines,[23] methylated guanosine and inosine

$$k' = k_0 + K_{zw}k_{\pm} \qquad (10)$$

derivatives,[22] and adenine nucleotides [1] support this viewpoint.

If the above interpretation is correct any structural modification which would have as its objective the blocking of the amino-group and/or its removal from near the imidazole ring should lead to important differences in the shape of the rate–pH profiles. This expectation is borne out in the results for *N*-acetylhistidine, glycyl-L-histidine, glycyl-L-histidylglycine, and β-alanylhistidine (Table 2 and Figure 2), the rate–pH profiles now reverting to the familiar bell-shape previously





witnessed for imidazole [4-7] and benzimidazole.[15] The relevant rate expression therefore is equation (11), leading to (12). The derived acidity and rate constants,

$$\text{Rate} = k[BH_2^+][OH^-] \qquad (11)$$

$$k_{obs.} = \frac{kK_w}{K_a + \dfrac{K_aK_a''}{[H^+]} + [H^+]} \qquad (12)$$

obtained in the usual way, are summarised in Table 3. The pK_a values are in good agreement with those obtained in the literature, once allowance for the temperature difference has been made *via* the Perrin equation. The results for the above-mentioned compounds, with the possible exception of glycyl-L-histidylglycine can clearly be described by a single curve with pK_a *ca.* 5.8 and pK_a'' *ca.* 12.7; for the tripeptide only a slightly lower pK_a (5.3) is required.

The above analysis is probably correct for *N*-acetylhistidine, where the amino-group has been blocked, but may be an over-simplification for the other peptides. A possible reason for this is suggested by the results for both histidine and histamine (Figure 1) where the first plateau is poorly defined especially when compared to some of the other compounds studied in this series, *e.g.* guanosine.[25] This probably stems from the closely similar pK_a' and pK_a values. A test of this hypothesis

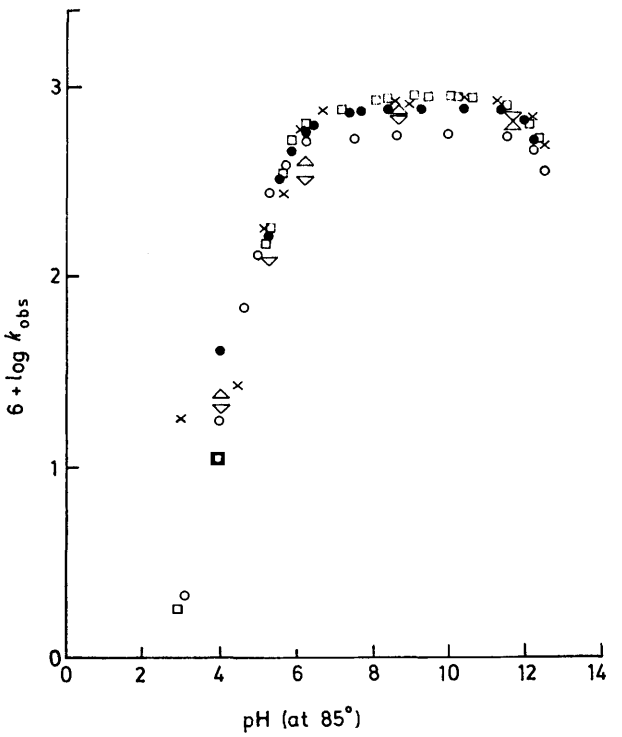


FIGURE 2 Rate–pH profiles for the detritiation of [2-^{3}H]-*N*-acetylhistidine and [2-^{3}H]histidine-containing peptides in aqueous buffers at 85°: (a) glycyl-L-histidine (×); (b) glycyl-L-histidylglycine (○); (c) *N*-acetylhistidine (□); (d) β-alanylhistidine (●); (e) histidylserine (△); (f) histidyltyrosine (▽)

can be readily carried out by using equation (7) to construct a number of theoretical rate–pH profiles. Thus in the case of histidine by using fixed pK_a (5.0) and k (6.1×10^3 l mol^{-1} s^{-1}) values and varying pK_a' we

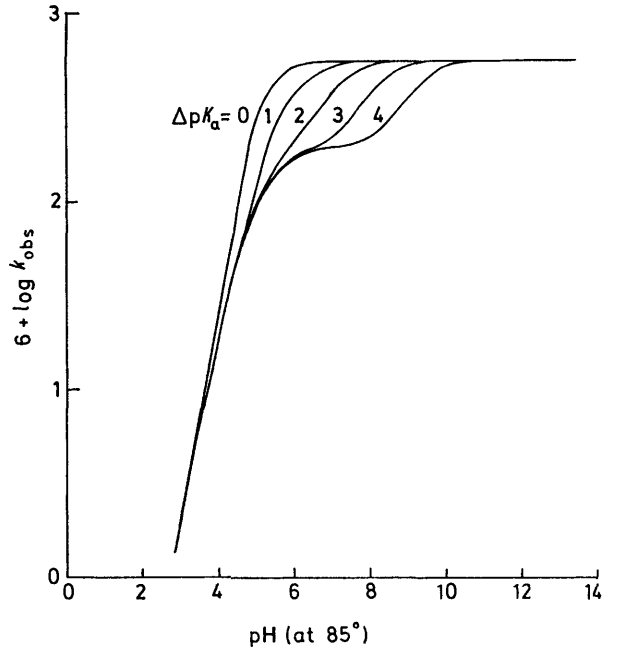


FIGURE 3 Theoretical rate–pH profiles for histidine using different ΔpK_a values

obtain Figure 3, showing clearly that only when $\Delta pK_a > 3$ are two clearly defined plateaus obtained and at lower values no clear discrimination between the two mechanisms is possible and a pseudo-bell-shaped rate–pH profile is obtained. The pK_a data in Table 3 shows that this is the situation that prevails for glycyl-L-histidine, glycyl-L-histidylglycine, and β-alanylhistidine and this is probably true for histidylserine and histidyltyrosine (Figure 2). Kinetic results (Table 2) for these two compounds are less extensive than for the other peptides. Kinetic and acidity data derived from fitting experimental data to theoretical rate–pH profiles must therefore be treated with caution. Whether reaction *via* the second pathway can still occur after removal of the protonated amino-group from the nearby imidazole ring can clearly best be tested with a substrate having $\Delta pK_a > 3$.

We are grateful to the S.R.C. for support of this work through a research studentship (S. E. T.) and a post-doctoral fellowship (M. S.).

[9/575 *Received*, 11*th April*, 1979]

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