Determination of p*K* Values of Peptide Groups in Dipeptides from Nuclear Magnetic Resonance Kinetic Studies

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The rate constants of the base catalysed exchange reaction, k_I, of dipeptides $RCONHCH_2CO_2^-$ with various halogen substituents on R were determined from n.m.r. line shape analysis of the methylene group of the glycyl residues. Changes in the values of k_I of the majority of the peptides were correlated successfully with proper substituent constants, determined from the dissociation constants of the corresponding carboxylic acids. By applying the Eigen scheme for proton transfer reactions we could explain the results obtained for all the peptides. Furthermore, it was possible to calculate absolute p*K* values of peptide groups from a knowledge of their k_I values. Measurements of the chemical shifts of the peptide hydrogens indicate the existence of a correlation between the chemical shifts of the peptide hydrogens and their relative acidity.

In a previous publication [1] a relative acidity scale for the peptide hydrogens of dipeptides, mainly of the form $NH_3^+[CH_2]_nCONHCH_2CO_2^-$ ($n = 1$—4), based on the changes of the rate constants of the base catalysed exchange reaction was proposed. The scale is defined by equation (1) where k_I is the rate constant of the base

$$\Delta \log k_I = \Delta \log (k_{I(RCONHCH_2CO_2^-)}/k_{I(CH_3CONHCH_2CO_2^-)}) \quad (1)$$

catalysed exchange reaction given by equation (2).

$$RCONHR' + OH^- \underset{}{\overset{k_I}{\rightleftharpoons}} RCON^-R' + H_2O \quad (2)$$

This definition of acidity and the actual determination from the kinetic data were chosen because no direct methods for determining the acidity of these groups is available. Measurements of the relative chemical shifts of the peptide hydrogens of the above series of dipeptides reveal the existence of a correlation between the relative shifts and the relative acidity.[2] While this work was in progress, Molday and Kallen [3] reported on the base catalysed exchange of amide protons of acyl-substituted amides.

In this work we have extended these studies, namely, we have measured the base catalysed exchange reaction and chemical shifts of peptide hydrogens of dipeptides $RCONHCH_2CO_2^-$ with various halogen substituents on R, *e.g.* β-chloro- and β-bromo-propionylglycine, chloro-, dichloro-, trichloro-, bromo-, and trifluoro-acetylglycine.

EXPERIMENTAL

The pH of the solutions was determined with a Radiometer pH meter type 26. The spectra were recorded on a Varian HA 100 spectrometer, using external locking capillaries. However, each sample contained traces of benzyl and t-butyl alcohols, which serve as an internal reference for the chemical shift determination.

Materials.—Chloroacetylglycine, bromoacetylglycine, and bromopropionylglycine were obtained from Miles, Yeda, Rehovoth, Israel. Trifluoroacetylglycine was prepared according to Weygand and Ropsch.[4] Dichloroacetylglycine, trichloroacetylglycine, and β-chloropropionylglycine were prepared by coupling glycine with the corresponding acid chlorides according to the following procedure. A small excess of the acid chloride was added dropwise to an aqueous solution of constant pH 9 at 5°. The solution was stirred for 1 h, acidified to pH 2, and extracted with ethyl acetate. The extract was dried (Na_2SO_4) and evaporated *in vacuo*. The product was crystallized from light petroleum to give crystals. The ninhydrin tests of the substances were negative. T.l.c. with n-butanol–acetic acid–water (1 : 1 : 1 or 25 : 6 : 25) showed one spot. The following m.p.s were obtained: dichloroacetylglycine 115, trichloroacetylglycine 117, and β-chloropropionylglycine 85°.

[1] M. Sheinblatt, *J. Amer. Chem. Soc.*, 1970, **92**, 2505.
[2] M. Sheinblatt, *J. Magnetic Resonance*, 1972, **8**, 55.
[3] R. S. Molday and R. G. Kallen, *J. Amer. Chem. Soc.*, 1972, **94**, 6739.
[4] F. Weygand and A. Ropsch, *Chem. Ber.*, 1959, **92**, 2095.

Calculation of Exchange Rates.—The n.m.r. spectra of the methylene group of the *C*-terminal glycine residue should be a doublet due to spin–spin interaction with the peptide hydrogen. (This pattern is observed in the spectra at low pH values.) As a result of the exchange of the peptide hydrogen, the doublet broadens and ultimately (for solutions in which the exchange is fast) coalesces into a single line. The mean lifetime (τ) between successive exchanges can be determined from line shape analysis of the methylene group of the *C*-terminal glycyl residue.[5] Since in the case of the β-halogenopropionylglycine the overlapping of the signals of the methylene groups of the glycyl and the propionyl groups prevent calculations of the rate of exchange over the entire pH range, some values of τ have been calculated from the broadening of the peptide hydrogen signals.

RESULTS

In Table 1 the reciprocal mean lifetimes of the peptide hydrogens of the different peptides as function of pH and concentration are given.

TABLE 1

Values of the reciprocal of the mean lifetime between successive exchanges ($1/\tau$) of the peptide hydrogens as function of pH and concentrations

Peptide	pH	$1/\tau$	pH	$1/\tau$	pH	$1/\tau$	pH	$1/\tau$
Trifluoroacetylglycine								
0·24M	4·13	7	4·65	9	4·95	12	5·29	19
0·32M	4·92	10	5·12	14	5·30	20	5·68	38
0·45M	4·65	9	5·02	13	5·34	20	5·50	28
Trichloroacetylglycine								
0·22M	5·28	8	5·43	10	5·69	13	5·87	16
	6·08	25	6·18	35	6·28	44		
0·39M	5·27	7	5·66	14	5·88	16	6·03	23
	6·10	28	6·21	37				
Dichloroacetylglycine								
0·35M	5·20	6	5·38	9	5·45	12	5·60	13
	5·70	14	5·76	15	5·96	25	6·24	44
	6·32	60						
0·25M	5·30	7	5·42	9	5·54	11	5·84	20
	6·12	42	6·33	55				
0·39M	5·22	6	5·49	10	5·91	27	6·11	41
Chloroacetylglycine								
0·24M	5·90	5	6·32	9	6·45	10	6·60	14
	6·73	15	7·14	31				
0·44M	6·40	8	6·50	9	6·90	18	7·15	28
	7·29	38	7·40	55				
Bromoacetylglycine								
0·68M	5·92	5	6·18	7	6·34	11	6·41	12
	6·55	14	6·65	17	6·93	25	6·94	28
	7·09	36						
0·34M	6·63	13	6·91	27	7·00	31	7·10	39
β-Chloropropionylglycine								
0·60M	7·37	5	7·80	10	8·11	16	8·22	22
0·72M	7·46	8	7·66	9	7·85	12	8·25	24
	8·38	26	8·50	35	8·63	47		
β-Bromopropionylglycine								
0·46M	7·54	5	7·71	8	7·86	10	8·00	14
	8·12	16	8·22	20	8·34	24	8·43	31
	8·60	43						
0·38M	7·88	9	8·00	13	8·16	16	8·23	19
	8·32	29	8·43	34	8·61	51		

In Table 2 the chemical shifts of the peptide hydrogens of the different molecules are summarized. The shifts refer to position of the spectral lines of the phenyl group of benzyl alcohol. Since the NH signal appears at lower field than that of the phenyl group, an increase in the chemical shift differences between the two lines indicates a low field shift of the NH signal.

TABLE 2

Chemical shifts of the spectral line of the peptide hydrogen referred to that of the phenyl group of traces of benzyl alcohol

Peptide	pH	Chemical shift (p.p.m.)
β-Bromopropionylglycine	7·6—8·5	0·71
β-Chloropropionylglycine	7·4—8·5	0·70
Bromoacetylglycine	5·4—6·5	0·96
Chloroacetylglycine	5·4—6·8	0·89
Dichloroacetylglycine	4·6—5·6	1·23
Trichloroacetylglycine	4·6—5·8	1·54
Trifluoroacetylglycine	4·6—5·4	1·90

DISCUSSION

Kinetic Measurements.—There are two possible exchange reactions for the peptide hydrogens,[6] (a) the base catalysed exchange reaction [defined in equation (2)] and (b) exchange with the solvent (water) molecules described by equation (3).

$$\mathrm{RCONHR' + H_2O \underset{}{\overset{k_{II}}{\rightleftharpoons}} RCON^-R' + H_3O^+} \quad (3)$$

The kinetic expression for these reactions in terms of the reciprocal mean lifetime of a peptide hydrogen between successive exchanges, $1/\tau$, is given by equation (4). According to equation (4) a plot of $1/\tau$ against $1/[H^+]$ should give a straight line with a slope of $k_I K_w$ and an intercept of $k_{II}[H_2O]$ independent of the peptide concentration. The experimental results summarized in Table 1 agree with this behaviour. A typical plot of the kinetic data for chloroacetylglycine is illustrated in Figure 1. Values of k_I and of $k_{II}[H_2O]$ were calculated from the experimental data using a standard least square procedure. The values are summarized in Table 3.

$$1/\tau = k_I[OH^-] + k_{II}[H_2O] = k_I K_w/[H^+] + k_{II}[H_2O] \quad (4)$$

TABLE 3

Values of the rate constants of the base catalysed reaction of the peptide hydrogens k_I and the exchange of the peptide hydrogens with solvent (water) molecules k_{II} $[H_2O]$

Peptide	k_I/l mol^{-1} s^{-1}	$k_{II}[H_2O]$/s^{-1}
Trifluoroacetylglycine	$7·0 \pm 0·3 \times 10^9$	$5·2 \pm 1·3$
Trichloroacetylglycine	$2·1 \pm 0·1 \times 10^9$	$2·7 \pm 0·9$
Dichloroacetylglycine	$2·6 \pm 0·1 \times 10^9$	$2·5 \pm 0·9$
Chloroacetylglycine	$1·9 \pm 0·1 \times 10^8$	$3·8 \pm 0·8$
Bromoacetylglycine	$2·6 \pm 0·1 \times 10^8$	$3·3 \pm 0·8$
β-Chloropropionylglycine	$9·9 \pm 0·4 \times 10^6$	$4·1 \pm 0·7$
β-Bromopropionylglycine	$1·1 \pm 0·1 \times 10^7$	$4·3 \pm 0·8$

The changes in the rate constants of the base catalysed exchange reaction arise from the presence of different

[5] A. Loewenstein and S. Meiboom, *J. Chem. Phys.*, 1957, **27**, 1067.

[6] A. Berger, A. Loewenstein, and S. Meiboom, *J. Amer. Chem. Soc.*, 1959, **81**, 62.

substituent groups in the peptide molecules. The substituent effect relative to that of acetylglycine can be

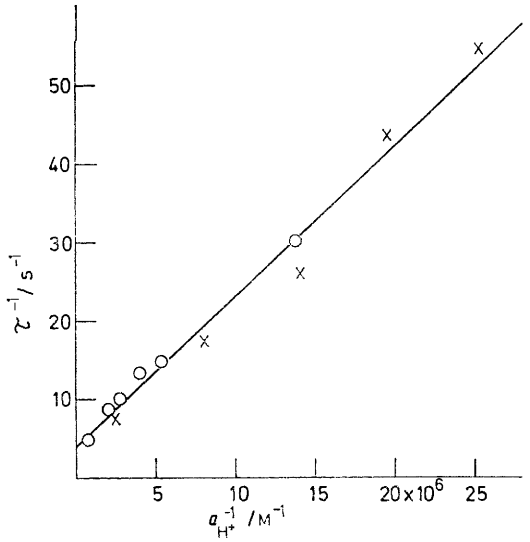


FIGURE 1 Values of the reciprocal mean lifetime between successive exchanges ($1/\tau$) of the peptide hydrogen of chloroacetylglycine (○ 0·24, × 0·44M) in aqueous solutions as a function of $1/a_{H^+}$

defined in terms of the kinetic data by log ($k_{I(RCOHHCH_2CO_2^-)}/k_{I(CH_3CONHCH_2CO_2^-)}$). This definition also represents a relative acidity scale ($\Delta \log k_I$) of the peptide hydrogens, since changes in the values of k_I can be looked upon as a measure of the relative tendency of the peptide groups to lose their protons to a common base molecule (OH^-). The effects of the same substituent groups determined from the changes of the dissociation constants of carboxylic acids,[7] log ($K_{RCO_2H}/K_{CH_3CO_2H}$), are correlated in Figure 2 with those derived from the kinetic studies.

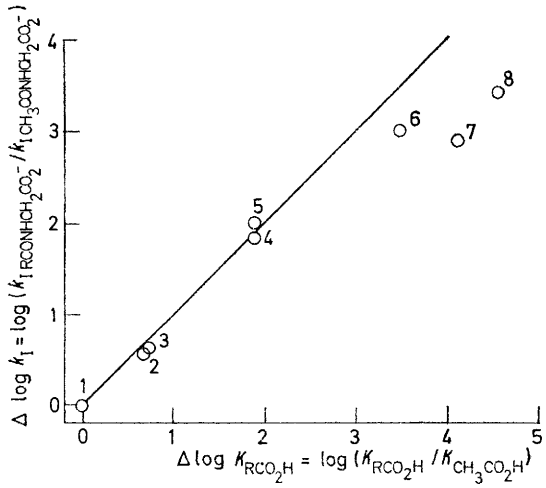
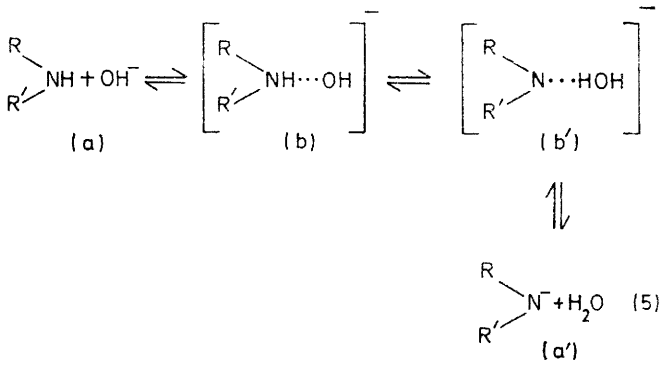


FIGURE 2 The relative acidity of the peptide hydrogens of dipeptides $RCONHCH_2CO_2^-$, $\Delta \log k_I$, as function of the relative acidity of carboxylic acids $\Delta \log K_{CO_2H}$: 1, R = Me; 2, R = $Cl(CH_2)_2$; 3, R = $Br(CH_2)_2$; 4, R = $ClCH_2$; 5, R = $BrCH_2$; 6, Cl_2CH_2; 7, R = CCl_3; 8, R = CF_3

(This definition of substituent effects unequivocally represents a relative acidity scale for carboxylic acids, $\Delta \log K_{CO_2H}$). It can be seen from Figure 2 that a linear free energy relationship, with a slope $\rho = 1$, exists between the two sets of substituent effects for the majority of the peptides, in excellent agreement with the same correlation observed previously for the charged dipeptides.[1] Deviation from linearity, which will be discussed later, is observed only for the very acidic peptides (R = CF_3 and CCl_3). The results reported by Molday and Kallen [3] show similar features, *i.e.* linear correlation, for the majority of the amides, between the rate constants of the base catalysed exchange reaction of the amide protons and pK_{CO_2H} (however with a slightly smaller slope $\rho = 0{\cdot}85$) and downward deviation for the most acidic trichloro- and trifluoro-derivatives. One may conclude from the above correlation that the changes in the rate constants of the base catalysed exchange reaction, $\Delta \log k_I$, measure the relative changes in the acidity of the peptide hydrogens as well as changes in $\Delta \log K_{CO_2H}$



measure the relative changes in acidity of the carboxylic acids.

Since the base catalysed exchange reaction describes a set of proton transfer reactions between a family of related donors (the peptides) and a common acceptor (OH^-) one can attempt to apply the Eigen formalism for such reactions [8] thus leading to reaction (5). With the usual approximations, the rate constant of the base catalysed exchange reaction can be written as (6) where

$$k_I = k_{ab}/(1 + 10^{-\Delta pK}) \qquad (6)$$

$\Delta pK = pK_{acceptor} - pK_{donor}$ and k_{ab} is the rate of formation of the encounter complex. Thus, depending on ΔpK, the Brønsted coefficient (α) should be (a) for $\Delta pK > 0$, $\alpha = 0$ (the reaction is diffusion controlled with a rate constant $k_I = k_{ab}$), (b) $\Delta pK < 0$, $\alpha = 1$ (k_I increases linearly with ΔpK), and (c) ΔpK *ca.* 0, α varied from 0 to 1 (the transition region).

On the basis of the previous conclusion that $\Delta \log K_{CO_2H}$ measures the changes in the acidity of peptide hydrogens, we may assume that $\Delta \log K_{CO_2H}$ equals $\Delta \log K_{NH}$ where K_{NH} is the dissociation constant of the peptide group and $\Delta \log K_{NH} = \log (K_{RCONHCH_2CO_2^-}/K_{CH_3CONHCH_2CO_2^-})$.

[7] The values of the pK_{CO_2H} were taken from W. P. Jencks and R. J. Regenstein, ' Ionization Constants of Acids and Bases,' in ' Handbook of Biochemistry,' ed. H. A. Sober, Chemical Rubber Co., Cleveland, 1970, 2nd edn.

[8] M. Eigen, *Angew. Chem. Internat. Edn.*, 1964, **3**, 1.

Accordingly, Figure 1 can be considered as representing a Brønsted plot with a linear plot ($\alpha = 1$) and a transition region ($0 \leqslant \alpha \leqslant 1$).

Taking into account our conclusion that the changes in the acidity of the peptide hydrogens equal those of the corresponding carboxylic acids, equation (6) can be re-written as (7) where ΔpK^* refers to acetylglycine and $a = \Delta pK_{CO_2H}$.

$$k_I = k_{ab}/(1 + 10^{-\Delta pK^* + a}) \qquad (7)$$

Considering the kinetic results presented here together with those published previously and treating the rate constant of the diffusion controlled base catalysed exchange reaction $k_{ab} = k_{max.}$ and ΔpK^* (that of acetylglycine) in equation (7) as two adjustable parameters, we

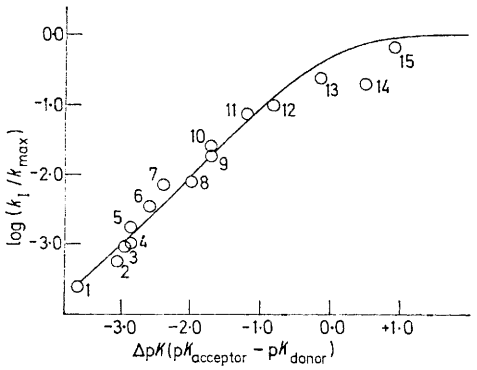


FIGURE 3 Eigen plot for proton transfer reaction [equation (7)] of the base catalysed exchange reaction of the peptide hydrogen of $RCONHCH_2CO_2^-$, log $k_I/k_{max.}$, as function of ΔpK: 1, R = Me; 2, R = $\overset{+}{N}H_3[CH_2]_4$; 3, R = $Cl(CH_2)_2$; 4, R = $Br(CH_2)_2$; 5, R = $\overset{+}{N}H_3[CH_2]_3$; 6, R = $\overset{+}{N}H_3CH_2CONHCH_2$; 7, R = $\overset{+}{N}H_3(CH_2)_2$; 8, R = H; 9, R = $ClCH_2$; 10, R = $BrCH_2$; 11, $\overset{+}{N}H_3CH_2$; 12, $\overset{+}{N}H_2C_4H_7$; 13, R = Cl_2CH; 14, R = CCl_3; 15, R = CF_3

obtained calculated values of log $k_I/k_{max.}$ as a function of ΔpK. These values are given in Figure 3 (solid line) and the values of the two adjustable parameters are $k_{max.} = 1{\cdot}1 \times 10^{10}$ l mol^{-1} s^{-1} and $\Delta pK^* = -3{\cdot}60$. The good agreement between calculated and experimental data indicates that (a) the changes in ΔpK_{NH} equals ΔpK_{CO_2H} and (b) the base catalysed exchange reaction of peptide hydrogens in dipeptides, and most probably also in other short chain peptides, can be described in terms of Eigen's scheme. Furthermore, it is possible, applying the Eigen formalism, to determine absolute pK_{NH} values of peptide hydrogens from a knowledge of kinetic data of their base catalysed exchange reaction. Calculated pK_{NH} values of typical peptides are summarized in Table 4. It seems, from Figure 3, that the accuracy of the calculated pK values for most of the peptides are within 0·2 pK units, while for the most acidic peptides the accuracy decreases. It should be remembered, however, that usually large deviations from the idealized Eigen behaviour are observed in the transition region. This may explain in part the results obtained for the very acidic peptides.

It is necessary also to consider the possibility that steric effects, such as bulky groups or formation of hydrogen bonds of the peptide hydrogens, may reduce the rate of exchange of the peptide hydrogens from their expected

TABLE 4

Typical calculated pK values of peptide groups of some peptide molecules

Peptide	pK
Acetylglycine	19·4
β-Bromopropionylglycine	18·6
Chloroacetylglycine	17·5
Bromoacetylglycine	17·5
Dichloroacetylglycine	15·9
Trifluoroacetylglycine	14·8
Glycylglycine	16·9
Glycylglycinamide	14·8
Glycylglycylglycine	17·9
Glycylglycylglycine	15·6
Prolylglycine	16·6
β-Alanlyglycine	18·1
γ-Aminobutyrylglycine	18·6

values. Thus, the relative large deviation of trichloroacetylglycine observed in Figure 3 and of *N*-methyltrichloroacetamide observed by Molday and Kallen [4] arises most probably from the bulky trichloromethyl group and does not suggest that their pK_{NH} values are higher than the values calculated on the basis of the substituent effect of the trichloromethyl group.

Chemical Shift Measurements.—The chemical shifts of peptide hydrogens are affected by changes in the rate of exchange of the hydrogens and by the state of ionization of ionizable centres in the peptide molecules. In order to eliminate these effects, chemical shift measurements were carried out for each peptide over a limited pH region in which the rates of exchange were low and the carboxy-groups of the glycyl residues were completely dissociated. Thus, we may attribute the observed relative shifts of the peptide hydrogens to the presence of the different substituent groups. The effect of these groups in terms of changes in the relative acidity of the peptide hydrogens

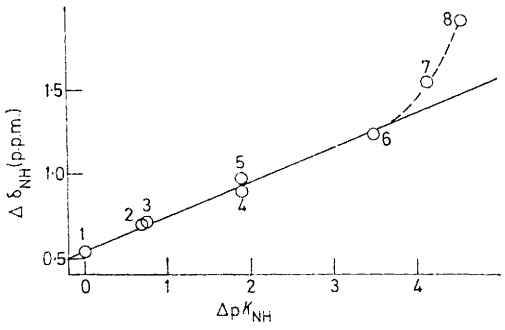


FIGURE 4 The relative chemical shifts of the peptide hydrogen signals $\Delta\delta_{NH}$ (referred to the signal of the phenyl group of benzyl alcohol) of $RCONHCH_2CO_2^-$ as function of the relative acidity of the peptide hydrogen ΔpK_{NH}: 1, R = Me; 2, R = $Cl(CH_2)_2$; 3, R = $Br(CH_2)_2$; 4, R = $ClCH_2$; 5, R = $BrCH_2$; 6, R = Cl_2CH; 7, R = CCl_3; 8, R = CF_3

ΔpK_{NH} are correlated in Figure 4 with their relative shifts. It can be seen that the more acidic the peptide group the more its NH signal shifts toward lower field.

A linear correlation between the two parameters is observed for peptides whose $\Delta pK_{NH} < 3{\cdot}5$ while the most acidic peptides, trichloro- and trifluoro-acetylglycine, exhibit an upward deviation. Similar results were reported by Molday and Kallen [4] for the shifts of amide protons dissolved in dimethyl sulphoxide.

The result that trichloroacetylglycine does not exhibit a downward deviation confirms our previous conclusion concerning the acidity of this peptide and the explanation proposed for the low value of its k_I.

Small shifts of the spectral lines of the methylene groups of the glycyl residues toward lower field with increasing electronegativity of the substituents were also observed. Thus, the shifts of these signals referred to that of t-butyl alcohol for bromopropionylglycine and dichloro-, trichloro- and trifluoro-acetylglycine are 254, 260, 263, and 264 Hz respectively.

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