Determination of an Acidity Scale for Peptide Hydrogens from Nuclear Magnetic Resonance Kinetic Studies

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Abstract: An attempt to determine the acidity of the peptide hydrogen in different peptide molecules from the studies of their base-catalyzed exchange reaction is discussed. The rates of the base-catalyzed exchange reaction of the peptide hydrogen of short peptide molecules have been investigated using the nmr technique. The exchange reaction is described by: $RCONHR' + OH^- \overset{k}{\rightleftharpoons} RCON^-R' + H_2O$. Values of the rate constant k change over 3 orders of magnitude among the different peptides that have been measured. An arbitrary acidity scale for the peptide hydrogens defined by $\Delta \log k = \log k_{RCONHCH_2COO^-}/k_{CH_3CONHCH_2COO^-}$ is proposed. Its validity has been proved from the existence of a linear free-energy relationship between $\Delta \log k$ and $\Delta \log K$ where K is the dissociation constant of carboxylic acids ($\log K_{RCOOH}/K_{CH_3COOH}$). The linear free-energy relationship is discussed also from various aspects. This discussion leads to conclusions concerning the nature of the exchange reaction, the hydration, and the formation of hydrogen bonds involving the peptide groups.

The acidity of peptide hydrogen, like the acidity of hydrogen atoms of other groups, probably depends on the nature and location of other functional groups present in the peptide molecule. Very little is known about the intrinsic acidity of the peptide hydrogen or the effects of substituent groups on the acidity of these hydrogen atoms. Knowledge about this property may be of importance in understanding the properties and behavior of peptide molecules such as the strength of the hydrogen bonds, CO···HN, found in α-helical structures of polypeptides and proteins.[2]

In this paper, an attempt to determine the acidity of the peptide hydrogen whose terminal amino group, NH_3^+, is located in various places in the molecules is described.

Since we are usually interested in studying the properties of the peptide group in aqueous media, it is natural that we would try to determine the acidity of the peptide hydrogen by its dissociation constant, K, *i.e.*

$$K = \frac{[RCON^-R'][H^+]}{[RCONHR']} \qquad (1)$$

However, because of the very low value of the dissociation constant of the peptide hydrogen, on the one hand, and the hydrolysis that the peptide molecules undergo in basic solution, on the other hand, direct methods for determining K are not applicable and one should look for an indirect method.

One of the indirect methods would be to determine the acidity of the peptide group from kinetic data, *i.e.*, to study a reaction which is identical for all peptide groups in the different peptide molecules, and which involves the release or gain of a proton by the peptide group.

Earlier publications[3] on the base-catalyzed exchange reaction of peptide hydrogen in aqueous solutions indicate that the above reaction can serve as a measure for the acidity determination of peptide hydrogens.

The exchange rates have been measured using the nmr technique which has the advantage that measurements are carried out in aqueous solution under mild conditions (pH 4–8). Thus the peptide molecules remain unaffected during the measurements.

In this work we report nmr measurements of the base-catalyzed exchange reaction of the peptide hydrogen of the following peptides: acetylglycine, β-alanylglycine, γ-aminobutyrylglycine, δ-aminovalerylglycine, formylglycine, prolylglycine, and glycylglycineamide. The kinetic data of the peptides mentioned above together with previous kinetic data,[3,4] besides having intrinsic importance, serve ultimately to build an acidity scale for the peptide hydrogen in the different molecules.

Base-Catalyzed Exchange Reaction of the Peptide Hydrogen

Berger, Lowenstein, and Meiboom[5] were the first to measure and to present quantitative data on the rate of exchange of a peptide hydrogen in aqueous solutions. Using the nmr technique, they measured the rate of exchange of the peptide hydrogen of N-methylacetamide (NMA) and found that the exchange reaction is catalyzed by the addition of either acid or base. The base-catalyzed exchange reaction of the peptide hydrogen of NMA is given by

$$CH_3CONHCH_3 + OH^- \overset{k}{\rightleftharpoons} CH_3CON^-CH_3 + H_2O \qquad (I)$$

The reaction is reversible, its equilibria lying almost completely to the left. The value of the rate constant of the forward reaction k was evaluated. Further measurements of the rate of exchange of peptide hydrogens of Gly-Gly[4] and Gly-Gly-Gly[3] have shown similar behavior, *i.e.*, the presence of a base-catalyzed exchange reaction which is identical with reaction I and can be represented similarly. The values of the rate constants for the base-catalyzed exchange reaction of these peptide hydrogens, however, are different: $k_{NMA} = 5.2 \times 10^6$ ($sec^{-1}M^{-1}$) while $k_{Gly\text{-}Gly} = 7.8 \times 10^8$ ($sec^{-1}M^{-1}$). The differences were attributed to

(1) (a) To where correspondence should be addressed. (b) Summer visitor at Varian Laboratory in 1966.
(2) G. L. Ling, *Biopolym. Symp.*, 1, 91 (1964).
(3) M. Sheinblatt, *J. Amer. Chem. Soc.*, 88, 2123 (1966).
(4) M. Sheinblatt, *ibid.*, 87, 572 (1965).
(5) A. Berger, A. Lowenstein, and S. Meiboom, *ibid.*, 81, 62 (1959).

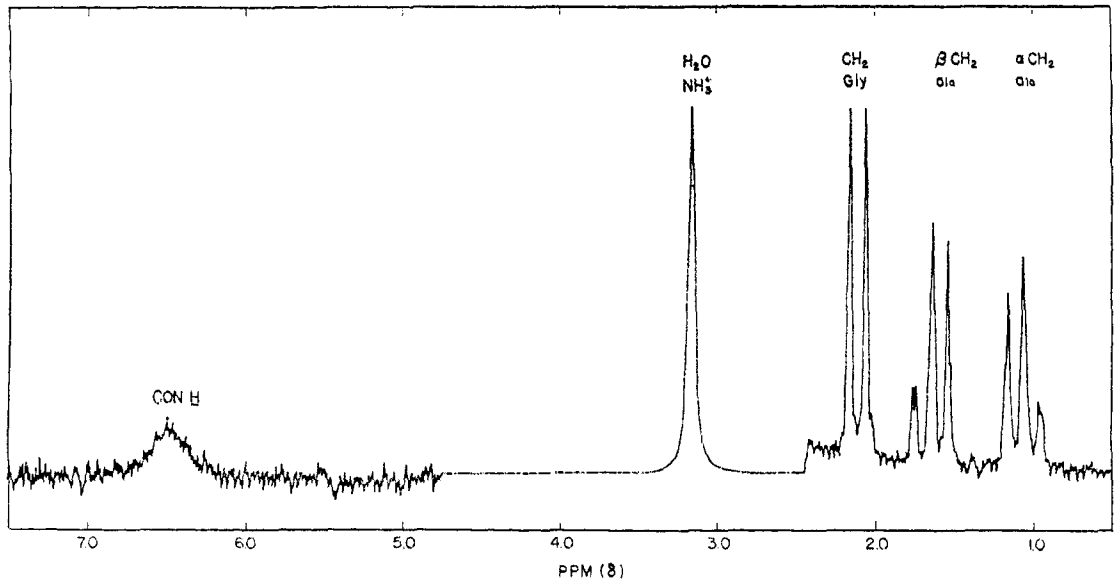

Figure 1. 60-MHz nmr spectrum of aqueous solution (pH 5.2) of β-Ala-Gly. The spectral lines have been recorded in different gains.

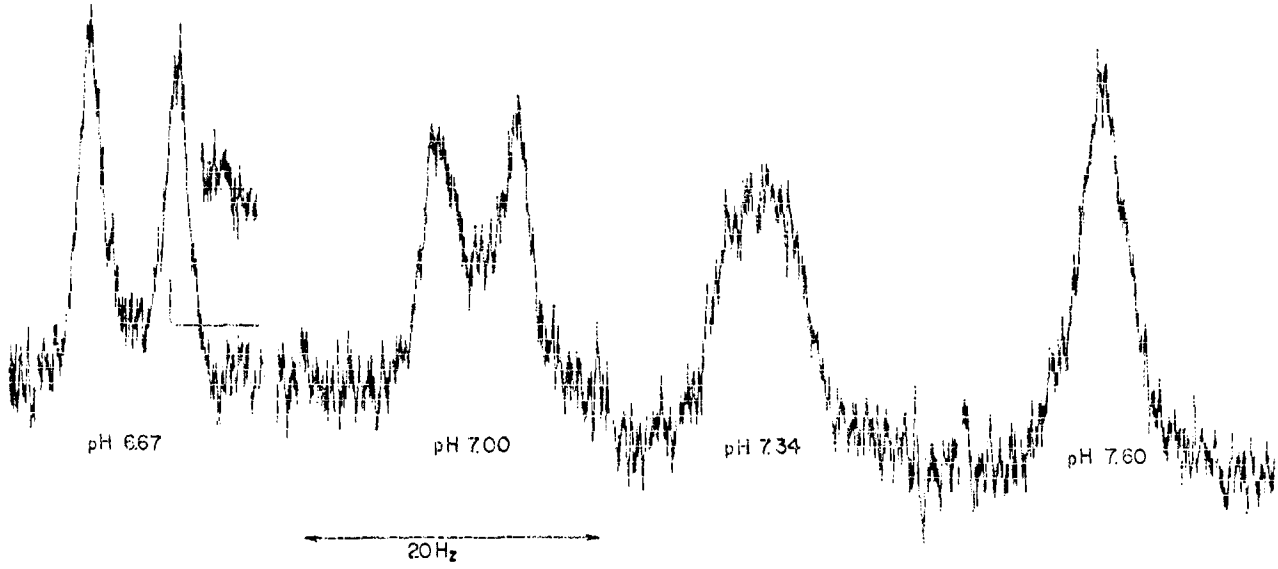

Figure 2. Typical spectral line shape of the methylene group of the glycine residue of β-Ala-Gly as a function of pH.

changes in the acidity of the peptide groups in the different peptide molecules which, in turn, arise from the presence of charged groups and the differences in the distances between the charged and peptide groups.[3]

Thus, the attempt to correlate the acidity of the peptide group with the rate constant of its base-catalyzed exchange reaction seems to be reasonable, provided that (i) the mechanism of the base catalyzed is identical for all groups and (ii) that the reaction is not diffusion controlled. The rate-determining step is the proton transfer from an acidic group, the peptide group, to a common base, OH^- ions.

The exchange reaction can then be described in a general form

$$RCONHR' + OH^- \underset{}{\overset{k}{\rightleftharpoons}} RCON^-R' + H_2O \qquad (IA)$$

Interpretation of the Nmr Spectra

Changes in the Nmr Spectra. Nmr spectra of aqueous solutions of β-alanylglycine, taken as a prototype for the peptide molecules under investigation, are described and discussed below.

In Figure 1, the nmr spectrum of an aqueous solution of β-Ala-Gly at pH 5.2 is shown. The lines in order of increasing magnetic field are: a broad line due to the peptide hydrogen; a sharp line due to the rapidly exchanging water and NH_3^+ protons; a doublet due to the methylene group of the glycine residue; and the two triplets due to the β and α methylene groups of the β-alanyl residue, respectively. Changes in the pH of the solution result in: (1) shifts of some spectral lines (illustrated in Table I) and (2) changes in the line shapes of the peptide hydrogen and of the methylene group of the glycine residue (illustrated in Figure 2). The shifts arise from changes in the ionization state of an ionizable center in the peptide molecule,[6] whereas the changes in the line shape are due to the changes in the rate of exchange of the peptide hydrogen.

(6) M. Sheinblatt, *J. Amer. Chem. Soc.*, 88, 2845 (1966).

Table I. Shifts in the Spectral Lines of β-Alanylglycine[a]

Group	Acid	Base
CH_2Gly	−18	
α-CH_2Ala	−5	+17
β-CH_2Ala	−2	+25
NH Peptide	−11	

[a] In basic and acidic solution as referred to their position at neutral pH, *i.e.*, when the peptide is in the zwitterion form. The + and − signs indicate up- and downshifts, respectively.

In the pH range where the rate of exchange of the peptide hydrogen is very slow, the NH signal appears as a broad single line. The multiplicity of the NH signal which is expected from its spin–spin interaction with the nitrogen and the methylene group of the glycine residue disappears as a result of the quadruple relaxation of the nitrogen atoms. Increase in the basicity of the solution results in broadening of the NH signal. This broadening can serve as a measure of the rate of exchange; however, the accuracy in this measurement is limited because of the NH signal width.

The spectral lines of the groups which are bound to the nitrogen atom of the peptide group, *i.e.*, the α-C groups of the amino acid residue, should be a doublet owing to the spin–spin interaction with the peptide hydrogen. As a result of the exchange of the peptide hydrogen, there is a change in the line shape which serves as a measure of the desired exchange.

Calculation of Rates of Exchange

The mean lifetime of a peptide hydrogen between successive exchanges can be determined from the line shape of the spectral line of the group bound to the nitrogen atom of the peptide group. The exact details of these calculations are discussed elsewhere.[4]

Experimental Section

Varian A-60, A-60-A, and AH-100 spectrometers with temperature-control V-6057 were used for the measurements. The temperature was 23 ± 2°. The pH of the solution was determined with a Radiometer pH-meter type 22r.

The peptides were of chromatographically pure grade and were obtained from the Yeda Research and Development Co. as well as from private sources.

Kinetic Results

The kinetic expression for reaction IA is

$$\frac{d[\text{peptide}]}{dt} = k[\text{peptide}][OH^-] \qquad (2)$$

Rewriting eq 2 in terms of the reciprocal mean lifetime of peptide hydrogen, $1/\tau$ and K_w, we obtain

$$\frac{1}{\tau} = \frac{kK_w}{[H^+]} \qquad (3)$$

According to eq 3 we would expect values of $1/\tau$ to be independent of the peptide concentration and linearly dependent on $1/[H^+]$.

The experimental results for the case of β-Ala-Gly are summarized in Figure 3. It is seen in this figure that values of $1/\tau$ are independent of the peptide concentration and depend linearly on $1/H^+$ as expected from eq 3.

Similar behavior was observed for all other peptide groups. The experimental results are summarized in Table II. From the slopes of the curves describing

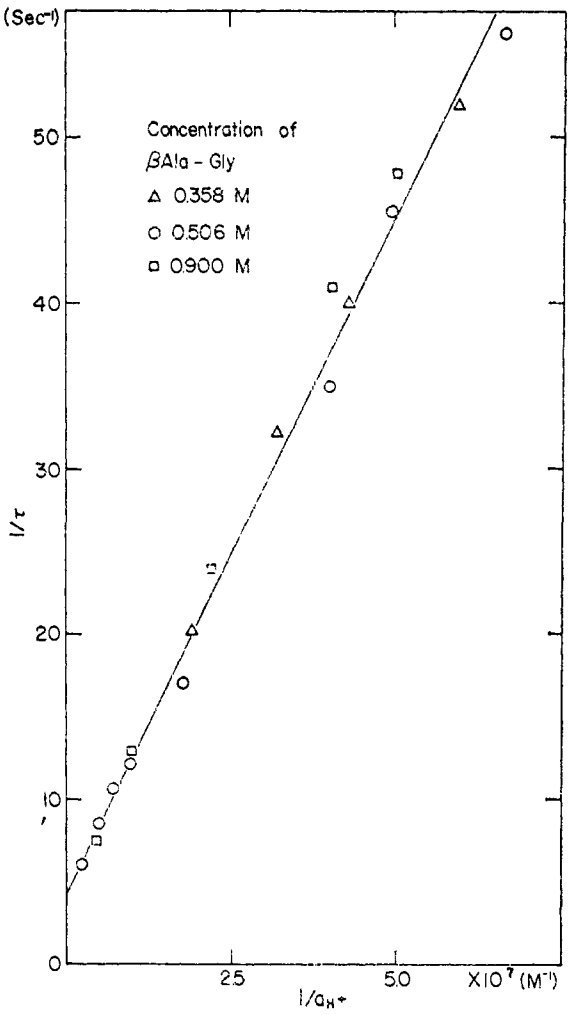

Figure 3. Typical plot for determining the rate constant of the base-catalyzed exchange reaction k. Values of the reciprocal of the mean lifetime $1/\tau$, of the peptide hydrogen of β-Ala-Gly for various peptide concentrations as a function of $1/a_{H^+}$.

$1/\tau$ as a function of $1/H^+$, the rate constants of the base-catalyzed exchange reaction, k, can be calculated. Values of k for the different peptide groups are summarized in Table III.

Discussion

We shall discuss various possibilities for determining the acidity of the peptide hydrogen from the kinetic data of the base-catalyzed exchange reaction. Although the different approaches for the acidity determination have much in common, we shall discuss them separately for purposes of convenience.

I. Dissociation Constant of the Peptide Group. The dissociation constant K (eq 1) of the peptide group can be expressed in terms of the rate constants of the base-catalyzed exchange reaction by

$$K = \frac{kK_w}{k'[H_2O]} \qquad (4)$$

where k and k' are the rate constants of the forward and backward base-catalyzed exchange reaction, respectively (mechanism IA). Since there are no available data concerning the reverse reaction, the dissociation constant K of the peptide group cannot be determined directly from eq 4. It is worthwhile, however, to

Table II. Values of the Reciprocal o f the Mean Lifetime between Successive Exchanges of the Peptide Protons ($1/\tau$)[a]

Peptide	pH	$1/\tau$	pH	$1/\tau$	pH	$1/\tau$	pH	$1/\tau$
Ac Gly								
0.45 *M*	8.15	9	8.22	9	8.49	12	8.59	16
	8.80	22	9.09	35	9.19	42		
0.76 *M*	8.10	7	8.30	10	8.50	14	8.63	17
	8.88	24						
β-Ala-Gly								
0.358 *M*	6.60	6	6.72	8	6.85	11	7.00	12
	7.25	17	7.60	35	7.69	45	7.82	56
0.506 *M*	7.28	20	7.50	32	7.63	40	7.77	52
0.900 *M*	6.67	7	7.00	13	7.34	24	7.60	41
	7.70	48						
γ-Am-But-Gly								
0.42 *M*	7.51	9	7.60	10	7.80	14	7.89	15
	8.13	27	8.24	32				
0.90 *M*	7.40	8	7.60	10	7.75	14	8.00	17
	8.14	24	8.14	29				
δ-Val-Gly								
0.48 *M*	7.40	4	8.00	8	8.17	11	8.72	31
	8.93	52						
0.33 *M*	8.03	7	8.18	9	8.36	16	8.60	28
	8.68	30	8.86	45				
0.46 *M*	7.90	6	8.10	8	8.30	13	8.60	27
	8.90	49						
Pro-Gly								
0.72 *M*	5.50	8	5.80	11	5.90	13	6.19	19
	6.25	24						
For-Gly								
0.28 *M*	7.12	6–7	7.38	10	7.58	15	7.75	23
	7.94	34	8.11	56				
0.60 *M*	7.00	6	7.12	8	7.73	23	7.84	33
Gly-Gly amide								
0.90 *M*	4.52	5	4.75	8	4.99	12	5.15	15
	5.35	26	5.48	40				
0.61 *M*	4.93	12	5.00	14	5.20	22	5.30	28
	5.50	42						

[a] In various peptide molecules as functions of pH and concentration.

Table III. Values of the Rate Constants of the Base-Catalyzed Exchange Reaction, k, for Various Peptide Molecules[d]

	Peptide	k, sec^{-1} M^{-1}
1.	$CH_3CONHCH_2COO^-$	2.7×10^6
2.	$NH_3^+CH_2CONHCH_2COO^-$ [a]	7.8×10^8
3.	$NH_3^+(CH_2)_2CONHCH_2COO^-$	8.0×10^7
4.	$NH_3^+(CH_2)_3CONHCH_2COO^-$	1.9×10^7
5.	$NH_3^+(CH_2)_4CONHCH_2COO^-$	6.2×10^6
6.	$NH_3^+CH_2CONHCH_2\underline{CONH}CH_2COO^-$ [b]	8.4×10^7
7.	$NH_3^+CH_2CONHCH_2\underline{CONH}CH_2COO^-$ [b]	6.7×10^9
8.	$NH_2^+C_4H_7\underline{CONH}CH_2COO^-$	1.1×10^9
9.	$NH_3^+CH_2CONHCH_2CONH_2$	1.0×10^{10}
10.	$HCONHCH_2COO^-$	3.8×10^7
11.	$CH_3CONHCH_3$ [c]	5.2×10^6

[a] Reference 4. [b] Reference 3. [c] Reference 5. [d] In the case of triglycine, we have underlined the corresponding peptide group.

emphasize that if the rate constants of the reverse reaction for the different peptides are almost equal, *e.g.*, if they are diffusion controlled, K would be proportional to k. In this case, changes in the values of k for the different peptides measure changes in the dissociation constant of the corresponding peptide groups.

II. Acidity Scale for the Peptide Hydrogen. The change in the rate constant of the base-catalyzed exchange reaction can be regarded as a change in the tendency of the peptide group, in the different molecules, to release its proton to a common base molecule, *i.e.*, the OH^- ion. Alternatively, the changes in k represent the ease with which an identical base molecule can remove the peptide proton from the different molecules. Both these approaches ultimately indicate changes in the acidity of the peptide hydrogen in the different molecules.

We have, however, to be careful in drawing conclusions since there might be other causes which can affect the rate of exchange without changing the acidity of the peptide hydrogen, such as the formation of intra- or intermolecular hydrogen bonds. However, as will be seen later, the effects originating from hydrogen bond formation are not important in the system that we have investigated. We shall endeavor, in the discussion below, to define the acidity of the peptide hydrogen based on our data in a quantitative manner.

(A) Proton Transfer Reaction. Proton transfer reaction can be described by a generalized Brønsted relationship d log k/d $\Delta pK = \alpha$ where the coefficient α is restricted to the values $0 \leqslant \alpha \leqslant 1$, k the rate constant of a proton transfer reaction, and $\Delta pK = pK$(acceptor) $- pK$(donor).

For the range: (i) $\Delta pK > 0$, $\alpha = 0$, k is independent of ΔpK (diffusion-controlled reaction); (ii) $\Delta pK < 0$, $\alpha = 1$, the rate constant k depends linearly on ΔpK; (iii) $\Delta pK \simeq 0$, the transition range, the value of α changes from unity to zero, *i.e.*, the values of k do not depend linearly on ΔpK.

However, for most of the donor–acceptor systems, we usually observe deviation from the idealized behavior. The deviation depends on the exact nature of the donor–acceptor systems.[7] The transition range may become very broad, over several ΔpK units. As a result, the linear dependence of log k and ΔpK is limited to a narrow range and occurs mainly for these acceptors in a donor–acceptor system for which $\Delta pK \ll 0$.

The kinetic results summarized in Table III indicate two main features: (i) changes of three orders of magnitude of the rate constants of the base-catalyzed exchange reaction; (ii) absence of a diffusion-controlled reaction, *i.e.*, we do not observe equally high values of k for two or more different peptides although the absolute value of k for some of the peptides is very high and probably differs by not more than one order of magnitude from an expected value of a diffusion-controlled base-catalyzed exchange reaction. This behavior excludes any reliable prediction on the exact correlation between k and ΔpK since the condition $\Delta pK \ll 0$, is hardly fulfilled in the system that we investigated.

(B) Substituent Effects. The most obvious factor responsible for the differences in the rate constants of the base-catalyzed exchange reaction, and hence for the difference in the acidity of peptide hydrogen, is the presence of various substituent groups among the different molecules. Let us define the substituent effect $\sigma = \Delta \log k = \log k_{RCONHCH_2COO^-}/k_{CH_3CONHCH_2COO^-}$[8] (according to the well-established Hammett ρ–σ relationship) and use it as an arbitrary measure of the acidity of peptide hydrogens.

The existence of a linear free-energy relationship between our substituent constants, defined above, and

(7) See *e.g.*, M. Eigen, *Angew. Chem. Intern. Ed. Engl.*, 3, 1 (1964).

(8) Since, in general, the R′ group of the peptide molecule RCONHR′ may also affect the rate of exchange of the peptide hydrogen, we have to consider only a series of peptides which have a common R′ group. We will thus consider only peptides of the type $RCOHNCH_2COO^-$.

substituent constants derived for different functional groups which measure, however, unequivocally changes in the acidity of the functional group, will confirm the validity of the proposed acidity scale for the peptide hydrogens.

We shall compare our substituent constants with substituent constants calculated from changes in the dissociation constants of substituted carboxylic acids: $\Delta\log K = \log K_{RCOOH}/K_{AcOH}$

In Figure 4 the two sets of the substituent constants for different R groups are summarized. This figure shows the existence of a linear correlation between the values of $\Delta\log k$ and $\Delta\log K$ over 3 logarithmic units. Furthermore, Figure 4 shows that the Hammett relationships for the two sets of substances derived from two quite different groups are equal to each other since $\rho = 1$.

This correlation indicates clearly that the effects of the substituent groups on the acidity of carboxylic groups are equal to the effects of the substituent groups on the rate of exchange of the peptide hydrogen. Thus, the acidity scale of the peptide hydrogen defined by $\Delta\log k$ seems to be a reliable measure for the relative acidity of the peptide hydrogens.

Figure 4 can be looked upon from a slightly different point of view. Accordingly, this figure describes a generalized Brønsted relationship for a proton transfer process described by the exchange reaction. Thus Figure 4 shows that $d\log k/d\,\Delta pK = \alpha = 1$. However, in this case ΔpK does not measure changes in the dissociation constants of the peptide groups, which participate in the exchange reaction, but rather changes in the dissociation constants of carboxylic groups which have nothing in common with the exchange reaction. This result seems to be strange unless we assume that $\Delta pK_{RCOOH} = \Delta pK_{RCONHR'}$. This equality confirms once more our previously defined acidity scale.

The fact that the exchange reaction of peptide hydrogens, including also these peptides with rate constants of 10^9, follows the idealized Brønsted relationship implies that (i) the reverse exchange reaction is diffusion controlled; (ii) values of the pK for the measured peptides are such that always $\Delta pK < 0$; (iii) the transition range (*i.e.*, the range in which the value of α changes from unity to zero) is relatively narrow.

It seems worthwhile to emphasize that the linear free-energy relationship enables us to calculate pK values of various peptide groups, provided that we know the dissociation constant of only one peptide group.

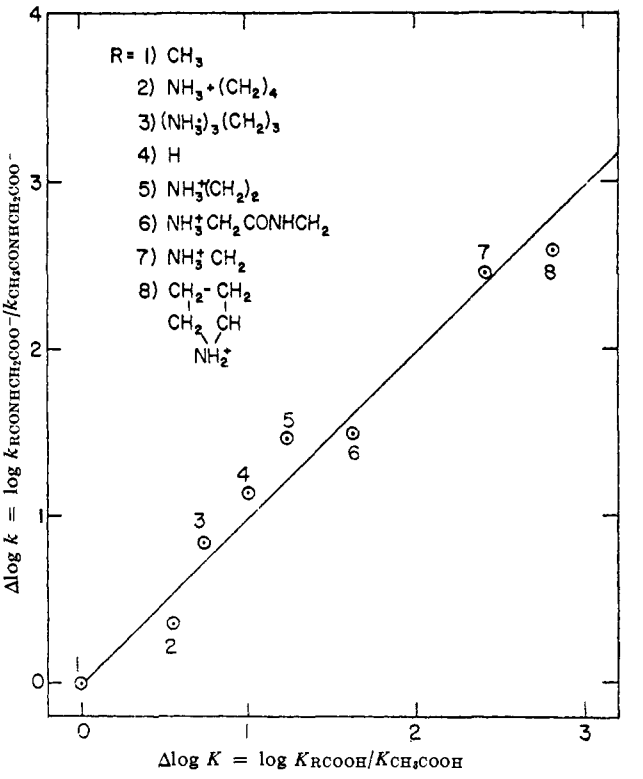

Figure 4. Values of the relative acidity of peptide hydrogen, as compared to that of Ac-Gly, $\log (k_{RCONHCH_2COO^-}/k_{CH_3CONHCH_2COO^-})$ as a function of the relative acidity of the corresponding carboxylic groups, $\log (K_{RCOOH}/K_{CH_3COOH})$ for various R groups.

We can estimate pK values of peptide groups from the above conclusions and the assumption that rate constants for a diffusion-controlled reaction for our exchange reaction are of the order of 10^{10}. Thus, the pK of the peptide group Gly-Gly is of the order $17 \leqslant pK \leqslant 18$.

Hydrogen Bonds. The formation of inter- (solvation) or intrahydrogen bonds involving the protons of the carboxylic or peptide groups should affect the acidity, or the rate of exchange of the two groups, respectively. The existence of the linear free-energy relationship indicates that the degree of inter- or intrahydrogen bonds of the protons of the two groups is equal. Moreover, because of the difference between the two groups, we may conclude that, most probably, there are no specific hydrogen bonds among the peptide groups in the different molecules.