CCXLVIII.—*The Action of Nitrous Acid on Amino-compounds. Part II. Aliphatic Amino-acids.*

By Thomas Weston Johns Taylor.

The action of nitrous acid on aliphatic amino-acids is largely used for their estimation by the method of van Slyke (*J. Biol. Chem.*, 1911, **9**, 275; 1912, **12**, 275); a large excess of nitrous acid is employed, and the progress of the reaction followed by measuring the volume of nitrogen evolved. Apart from qualitative estimates of the relative speeds of reaction of various acids, however, no measurements of reaction velocity have been made, with the exception of the work of Dunn and Schmidt (*ibid.*, 1922, **53**, 401), who, using van Slyke's method, investigated a series of acids and showed that the reaction was more rapid the nearer the amino- and the carboxyl group were in the carbon chain. They also state that the amino-acid disappears at a rate in accordance with the unimolecular law, although their experimental results are far from consistent.

The amino-acids react with nitrous acid much more rapidly than do methylamine and ammonia, and in order to throw light on this difference, measurements of the reaction velocity in the cases of glycine, α-alanine, and β-alanine have been made. For reasons pointed out in Part I (this vol., p. 1099) any method similar to that of van Slyke is unsuitable, and the reaction has been followed by estimation of the amino-acid. It is shown that the difference referred to arises from the fact that, with an amino-acid $NH_2 \cdot R \cdot CO \cdot OH$, the form $NH_3^+ \cdot R \cdot CO \cdot O^-$ is able to react rapidly.

Method and Materials.—The reactions were investigated at $25° \pm 0.1°$. The solutions were made up from standard solutions of the amino-acid, of sulphuric acid and of pure barium nitrite; the mixture was protected by a layer of medicinal paraffin (see Part I),

through which passed a small stirrer and a tube; through the latter, samples were withdrawn from time to time by pipette.

The sample was immediately run into excess of aqueous baryta and the amino-acid content estimated by a modification of Sörensen's formaldehyde titration method (*Biochem. Z.*, 1908, **7**, 45). Phenolphthalein was added and dilute sulphuric acid run in from a burette until the pink colour just disappeared; excess of 20% formaldehyde solution, which had been previously neutralised to phenolphthalein by baryta, was then added and the amino-acid was titrated with 0·05N-baryta. The method was found by blank experiments to give results accurate to 1% in the case of glycine, if the final concentration of the glycine was not less than about 0·01N; with more dilute solutions the end-point is not very definite. In the case of the two aminopropionic acids the end-point is not so good and the accuracy consequently suffers; no other method giving a greater accuracy is, however, available.

The glycine was a commercial A.R. sample which had been recrystallised three times from aqueous alcohol. α-Alanine was prepared by the method of Zelinsky and Stadnikoff (*Ber.*, 1908, **41**, 2061) and β-alanine from succinimide (Holm, *Arch. Pharm.*, 1904, **242**, 597); after three recrystallisations from aqueous alcohol the α-compound melted at 295° (corr.) and the β at 206° (corr.); the latter melting point is 10° higher than that usually accepted, but agrees with the observation of Lengfeld and Stieglitz (*Amer. Chem. J.*, 1893, **15**, 508).

Results.

The three acids show qualitatively the same behaviour towards nitrous acid. At a dilution of 0·05N with an equivalent of barium nitrite no reaction could be detected over a period of 6 hours. With an equivalent of nitrous acid there is a rapid reaction unlike the cases of methylamine and ammonia; the rate of this reaction is lowered by the addition of mineral acids or of neutral salts.

Glycine.—Curves I and II of Fig. 1 show the rate of reaction with an equivalent of nitrous acid in 0·05N- and 0·025N-solution respectively. The reaction is so fast that nitrogen bubbles freely from the solution and thus the spontaneous decomposition of the nitrous acid is pronounced; consequently the interpretation of the results is not so simple as in the case of methylamine (Part I, *loc. cit.*). Since the velocity is lowered by the presence of sulphuric acid (Curves III and IV, Fig. 1) and since a product of the reaction is glycollic acid, experiments were carried out with solutions to which glycollic acid had been added (Curve I, Fig. 1). Even if this is in large excess (0·25N), however, the velocity of reaction is unaffected, and thus there is no negative autocatalysis.

The reaction with one equivalent of nitrous acid is approximately of the third order. From the smooth curves shown in Fig. 1 values of the glycine concentration at intervals of 10 minutes were read and from these termolecular velocity coefficients were calculated; these are shown in Table I for both 0·05N- and 0·025N-solutions; k cannot be expected to remain constant because of the marked

FIG. 1.
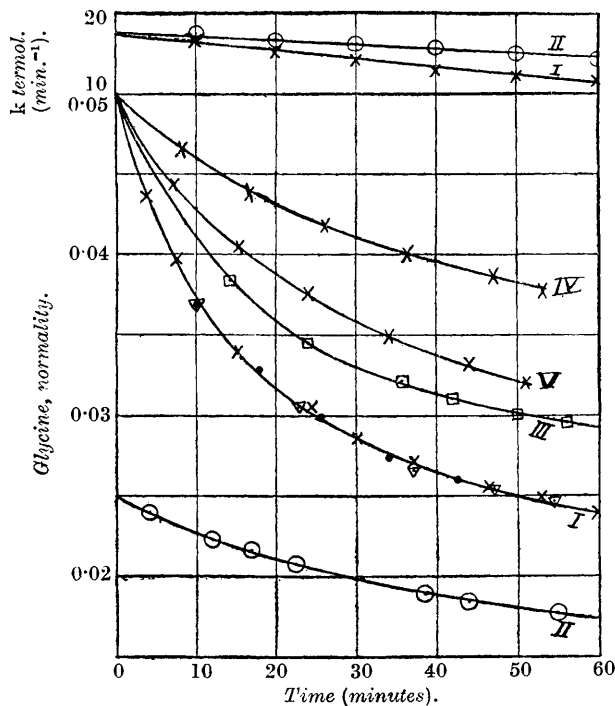
1. × 0·05N-*Glycine* + 0·05N-HNO$_2$.
   •   ,,   +   ,,   + 0·05N-*glycollic acid.*
   ▽   ,,   +   ,,   + 0·25N-   ,,
II. 0·025N-*Glycine* + 0·025N-HNO$_2$.
III. 0·05N-*Glycine* + 0·05N·HNO$_2$ + 0·05N-H$_2$SO$_4$.
IV.   ,,   +   ,,   + 0·1N-   ,,
V.   ,,   +   ,,   + 1·0N-KCl.

spontaneous decomposition of one of the reactants. If, however, these values are plotted against time, as in the upper part of Fig. 1, and straight lines are drawn through them, the lines for the two dilutions converge to the same value at time 0.

The values of k fall more rapidly for the stronger solution, as would be expected, since the rate of spontaneous decomposition of the nitrous acid is greater in that solution because of the higher rate

TABLE I.

Glycine.

| | 0·05N-Soln. | | | 0·025N-Soln. | |
min.	x norm.	k termol. (min.$^{-1}$).		x norm.	k termol. (min.$^{-1}$).
0	0·05	—		0·025	—
10	0·0372	16·2		0·0227	17·0
20	0·0317	14·9		0·0208	17·0
30	0·0285	13·9		0·0198	15·9
40	0·0265	12·8		0·0188	15·4
50	0·0250	12·0		0·0180	14·9
60	0·0238	11·4		0·0174	14·2

FIG. 2.

Time (minutes).
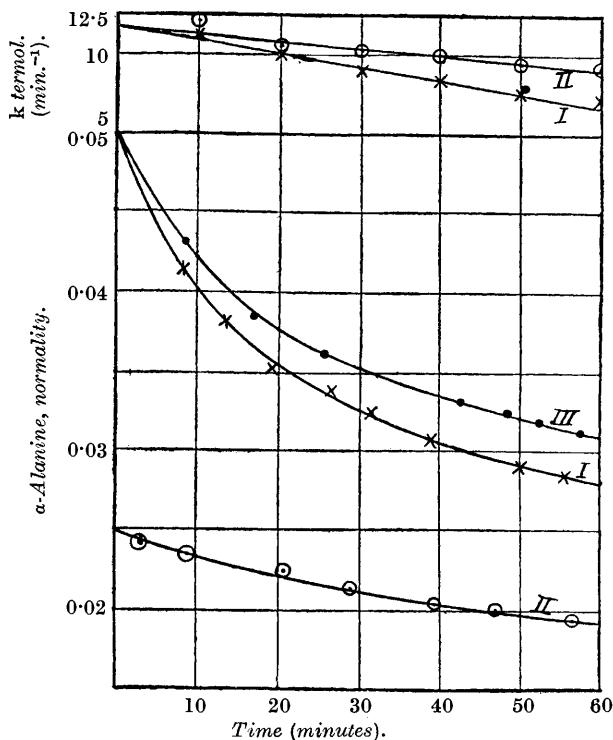
I. 0·05N-α-*Alanine* + 0·05N-HNO$_2$.
II. 0·025N-   ,,   + 0·025N- ,,
III. 0·05N-   ,,   + 0·05N- ,,   + 0·5N-KCl.

of passage of gas bubbles through the liquid. If any other simple order is assumed for the reaction, there is no kind of convergence of the k values; assuming a quadrimolecular reaction, which gives more constant values for k at any one dilution, the extrapolated

value for k at 0·05N is 390 min.$^{-1}$, and at 0·025N 720 min.$^{-1}$; and for a bimolecular reaction 0·78 at 0·05N and 0·43 at 0·025N.

Experiment showed that doubling the concentration of nitrous acid increased the rate of reaction much more than doubling the concentration of glycine. Hence of the three reacting molecular species, one must be glycine in some form and the other two nitrous acid; there is an immediate analogy with the case of methylamine, where the reaction velocity is proportional to the product $[RNH_3^{\cdot}][NO_2'][HNO_2]$.

α-*Alanine and* β-*Alanine.*—Precisely the same holds for these two acids, except that β-alanine reacts much less readily, so that in 0·025N-solution the velocity is too slow for accurate measurement. Measurements were therefore made in 0·05N- and 0·04N-solution. The results are shown in Figs. 2 and 3 and in Tables II and III.

TABLE II.

α-Alanine.

	0·05N-Soln.		0·025N-Soln.	
t min.	x norm.	k termol. (min.$^{-1}$).	x norm.	k termol. (min.$^{-1}$).
0	0·05	—	0·025	—
10	0·0401	11·1	0·0233	12·1
20	0·0354	10·0	0·0222	10·7
30	0·0326	9·0	0·0212	10·4
40	0·0306	8·4	0·0204	10·0
50	0·0290	7·6	0·0198	9·5
60	0·0279	7·4	0·0193	9·4

TABLE III.

β-Alanine.

	0·05N-Soln.		0·04N-Soln.	
t min.	x norm.	k termol. (min.$^{-1}$).	x norm.	k termol. (min.$^{-1}$).
0	0·05	—	0·04	—
10	0·0460	3·33	0·0380	3·40
20	0·0435	3·10	0·0364	3·25
30	0·0418	2·87	0·0350	3·16
40	0·0402	2·74	0·0339	3·07
50	0·0388	2·65	0·0329	2·99
60	0·0375	2·60	0·0321	2·88

Now an aliphatic amino-acid, $NH_2 \cdot R \cdot CO \cdot OH$, in solution together with the weak acid nitrous acid exists almost entirely in the two forms $NH_3^+ \cdot R \cdot CO \cdot O^-$ (A^{+-}) and $NH_3^+ \cdot R \cdot CO \cdot OH$ (A$^+$) (Bjerrum, *Z. physikal. Chem.*, 1923, **104**, 147). If the total concentration of the amino-acid is equal to that of the nitrous acid and is x, and if a fraction f of the acid is present as the ion A$^+$, we have

$$(1 - f)x[H^{\cdot}]/fx = K_8 \quad . \quad . \quad . \quad . \quad (1)$$

where K_8 is the acid dissociation constant of the amino-acid and

3 R

[H$^{\bullet}$] is the hydrogen-ion concentration.   Further, since the whole solution is electrically neutral, $[NO_2'] = [H^{\bullet}] + [A^+]$, which gives
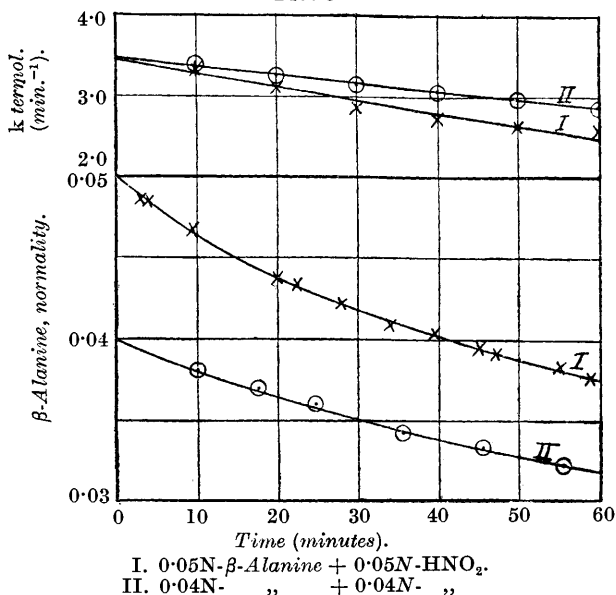
$$[NO_2'] = fx + [H^{\bullet}] \text{ and } [HNO_2] = (1 - f)x - [H^{\bullet}] . \quad . \quad (2)$$

Therefore, if K_N is the dissociation constant of nitrous acid,

$$(fx + [H^{\bullet}])[H^{\bullet}]/\{(1 - f)x - [H^{\bullet}]\} = K_N \quad . \quad . \quad (3)$$

Elimination of [H$^{\bullet}$] between equations (1) and (3) leads to a cubic equation connecting x and f.   Numerical values were substituted for K_s, K_N and x, giving to the last the values $0.05N$ and $0.025N$

FIG. 3.

I. $0.05N$-β-Alanine + $0.05N$-HNO_2.
II. $0.04N$-   ,,   + $0.04N$-   ,,

successively, and values for $(1 - f)$ were calculated; these are shown in Table IV.

TABLE IV.

	K_s.	$(1-f)$, $0.05N$.	$(1-f)$, $0.025N$.	% Change.
Glycine	4.7×10^{-3}*	0.786	0.800	1.75
α-Alanine	2.45×10^{-3}*	0.721	0.718	0.43
β-Alanine	2.49×10^{-4}†	0.444	0.444	0

* Bjerrum, loc. cit.
† Calculated from Ley's results (Ber., 1909, **42**, 374).

It can be seen that the fraction of the amino-acid present in either form hardly changes in value on alteration in concentration, and the assumption is made here that it is independent of concentration.
    The form of the amino-acid which reacts with nitrous acid may be

A^+ or A^{+-} or both. Now addition of sulphuric acid lowers the reaction velocity; if A^+ is the reacting form, the concentration of this form will be increased by addition of mineral acid, and hence the nitrite ion must be involved in the reaction, since there is no other derivative of nitrous acid whose concentration is decreased by mineral acid.

If, however, A^{+-} is the reacting form, the concentration of this is decreased by increasing the hydrogen-ion concentration (compare equation 1), and thus the velocity may be proportional to the product $[A^{+-}][HNO_2]^2$. The measurement of reaction velocity in the presence of sulphuric acid shows that of all possible hypotheses the latter is correct.

The results of measurements with $0.05N$-glycine and $0.05N$-nitrous acid in the presence of $0.05N$-sulphuric acid and of $0.1N$-sulphuric acid are shown in curves III and IV of Fig. 1. Assuming that the reaction velocity is proportional to the product $[A^{+-}][HNO_2]^2$, let the concentration of glycine and of nitrous acid be a initially and x at time t. For the results shown in curve IV the amount of sulphuric acid present is $2a$; the hydrogen-ion concentration at time t may be taken as $2a-x$, since the glycine will be almost completely in the form A^+. Hence, if f is the fraction of glycine present as A^+, we have analogously with equation 1,

$$(1-f)x(2a-x)/fx = K_8,$$

whence $f = (2a-x)/(K_8 + 2a - x)$. It can be further assumed that the nitrous acid is completely undissociated in the presence of the sulphuric acid, and thus on the above assumption we get for the reaction velocity

$$-dx/dt = kx^3 K_8/(K_8 + 2a - x),$$

which on integration between times t and 0 gives

$$k = \frac{1}{t}\left(\frac{a^2 - x^2}{2a^2 x^2} + \frac{a - x}{K_8 x^2}\right).$$

Values of k calculated from this equation for every 10 minutes are shown in Table V.

For the reaction in the presence of one equivalent of sulphuric acid, after a certain time an appreciable amount of free sulphuric acid is present in the solution, since some glycine has disappeared. The hydrogen-ion concentration can be taken as equal to that of this free acid; thus at time t, $[H^{\cdot}] = (a - x)$. In a fashion similar to that above, it can be shown that

$$-dx/dt = kx^3 K_8/(K_8 + a - x).$$

This expression cannot be integrated back to the beginning, since

the assumptions made do not hold at the start of the reaction. Integration between times t_2 and t_1 gives

$$k = \frac{1}{t_2 - t_1}\left[\frac{K_8 + a}{2K_8 x^2} - \frac{1}{K_8 x}\right]_{x_1}^{x_2}.$$

Values of k calculated from this with $t_1 = 20$ mins. are shown in Table V.

Now for the reaction between glycine and nitrous acid alone, the fraction of glycine present in the form A^{+-} is given in Table IV; the concentration of undissociated nitrous acid is given by equation (2), since the term [H˙] can be obtained from equation (1). Thus on the present assumption the reaction velocity should be given by

$$-\frac{dx}{dt} = k(1-f)x\left(\overline{1-f}x - \frac{K_8 f}{1-f}\right)^2$$

whence, assuming f is independent of concentration and writing $(1-f) = b$ and $K_8 f/(1-f) = c$,

$$k = \frac{1}{bct}\left(\frac{1}{bx - c} - \frac{1}{ab - c} - \log_e \frac{x}{bx - c} \cdot \frac{ab - c}{a}\right) . \quad . \quad (4)$$

Values of k calculated both for $0.05N$- and $0.025N$-solutions are shown in Table V.

It will be seen that the values of k from the four sets of experiments agree as well as could be expected. In the reactions which proceed more slowly, and thus where the spontaneous decomposition of the nitrous acid is less marked, the values of k are higher and fall off less rapidly.

TABLE V.

Glycine.

| | 0.05N-Glycine + 0.05N-HNO$_2$. | | | | 0.025N-Glycine + 0.025N-HNO$_2$. |
| | 0.1N-H$_2$SO$_4$. | | 0.05N-H$_2$SO$_4$. | | Alone. | |
t.	x.	k.	x.	k.	k.	k.
0	0·0500	—	0·0500	—	—	—
10	0·0459	45·1	0·0411	—	39·2	45·0
20	0·0430	44·2	0·0357	—	33·5	40·3
30	0·0410	41·1	0·0332	32·8	29·4	37·5
40	0·0395	38·8	0·0311	28·5	27·9	34·2
50	0·0382	35·8	0·0300	25·7	26·5	33·1
	(Curve IV.)		(Curve III.)		(Curve I.)	(Curve II.)

If any other assumption is made as to the molecular species involved in the reaction, similar calculations can be carried out, and it is found that there is no kind of agreement between the values derived from the four sets of experiments.

Taking it as proved that the reaction velocity is proportional to the product $[A^{+-}][HNO_2]^2$, values for the true velocity coefficients for the three acids can be obtained from (4), plotted against time

and extrapolated to zero time. The results are in Table VI; they are not very accurate for the alanines because equation (4) is very sensitive to small changes in x, and the analytical method is not capable of the accuracy it demands.

TABLE VI.

	k (min.$^{-1}$).	K_8.
Glycine	46	4.7×10^{-3}
α-Alanine....................................	32	2.45×10^{-3}
β-Alanine	45	2.49×10^{-4}

The apparent difference in the speeds of reaction in the three cases arises mainly from the difference in the acid dissociation constants K_8. A small value of K_8 causes a small concentration of the form A^{+-} and therefore a small reaction velocity. The results of Dunn and Schmidt (loc. cit.) are thus in agreement with the general conclusions of Bjerrum (loc. cit.), that the more closely the amino- and the carboxyl group are situated in the carbon chain, the greater the value of K_8.

The possibility remains that the reaction may also proceed by an alternative mechanism, at a rate proportional to the product $[A^+][NO_2'][HNO_2]$ as in the case of methylamine and ammonia. If the velocity coefficient for this mechanism is of the same order as in those two cases, 1 min.$^{-1}$, the effect of this mechanism will be small compared with the " Zwitterion " mechanism, and the experimental accuracy is not great enough to detect it.

The results arrived at have two possible interpretations. Either the reaction is actually of the third order, or else it is a reaction between undissociated nitrous acid and a complex formed from the " Zwitterion " and nitrous acid, since the concentration of any such complex will be proportional to the product $[A^{+-}][HNO_2]$. Taking into account the results with methylamine, the latter explanation becomes the more probable; an uncharged complex can only arise in the case of methylamine from the molecular species $CH_3 \cdot NH_3^+$ and NO_2'; in the amino-acids it can arise from the species A^{+-} and HNO_2; and in each case the reaction velocity is proportional to the product of the concentration of the appropriate molecular species multiplied by that of undissociated nitrous acid. The velocity coefficient is forty times larger in the amino-acids than in the amine; the difference, on this view, would be a measure of the ratio of the probabilities of the formation of a complex from the species A^{+-} and HNO_2 and from the ions RNH_3^+ and NO_2'.

Effect of Neutral Salts.

The effect of neutral salts is of interest because of the view of Pfeiffer that neutral salts and amino-acids form complexes which

exist in solution as well as in the solid state (references in Pfeiffer's " Organische Molekulverbindungen," 1927, p. 143 *et seq.*).   His results indicate that calcium chloride is more able to form complexes than potassium chloride; but the reaction velocities in the presence of $0.05N$-calcium chloride and of $0.05N$-potassium chloride were found to be the same within the limits of error of the method.   The retarding effect of neutral salts is also found in the cases of methyl-amine and ammonia, where it is almost certainly an ionic strength effect, and thus the present results provide no support for Pfeiffer's views.

Summary.

1. The velocity of reaction of nitrous acid with glycine, α-alanine, and β-alanine has been measured at 25° in dilute aqueous solution.

2. The reaction is approximately of the third order, and is retarded by the presence of neutral salts or of mineral acid.

3. It is shown that the principal reacting species are undissociated nitrous acid and the amino-acid in its " Zwitterion " form $NH_3^+ \cdot R \cdot CO \cdot O^-$.   The implications of this result are discussed.