32. *The Deamination of Amino-acids by Nitrous Acid with Particular Reference to Glycine. The Chemistry underlying the Van Slyke Determination of α-Amino-acids.*

By A. T. Austin.

The deamination of α-amino-acids by nitrous acid, which is the basis of the Van Slyke gasometric method for the determination of amino-acids, is not, as is generally considered, accurately described (for the example of glycine) by the equation :

$$H_3N^+{\cdot}CH_2{\cdot}CO_2^- + HNO_2 \longrightarrow HO{\cdot}CH_2{\cdot}CO_2H + N_2 + H_2O$$

In the case of glycine the gas evolved is a mixture of nitrogen, carbon dioxide, and nitrous oxide, and increases in volume with increasing nitrite-ion concentration. There is a corresponding decrease in the amount of glycollic acid in the reaction residue, and from this residue nitromethane and methyl nitrolic acid have been isolated. These facts are explained by the assumption that in the deamination process an intermediate is formed which can interact with negative ions, such as chloride or nitrite ions (giving α-substituted acids), or with water molecules (giving α-hydroxy-acids). By nitrite-ion interaction it appears that α-nitroacetic acid is formed in the above reaction, and it has been shown that this acid may either decompose to nitromethane and carbon dioxide or react with nitrous acid to give methylnitrolic acid and carbon dioxide. At pH 4—5 methylnitrolic acid is converted into a polymer of fulminic acid with much frothing. This polymer breaks down to formohydroxamic acid, and it is shown that, under the above experimental conditions, 10% of this decomposes to cyanic acid and 90% to formic acid and hydroxylamine. The latter with nitrous acid gives nitrous oxide. An explanation is thus obtained for the anomalous gas volumes and troublesome frothing encountered in the Van Slyke nitrous acid determination of glycine.

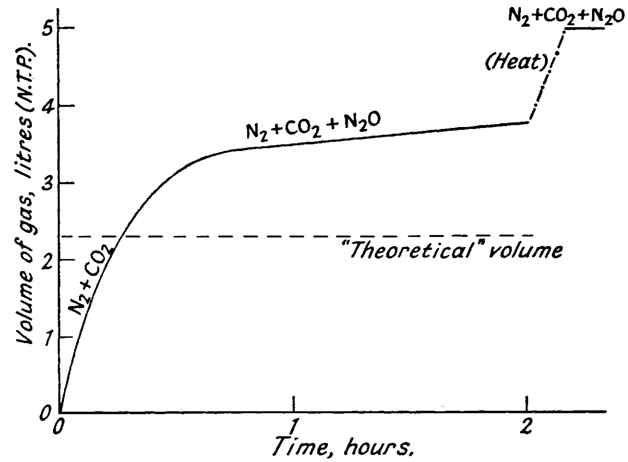
The analytical determination of aliphatic amino-groups by the reaction with nitrous acid and measurement of the volume of nitrogen liberated presents an anomaly with α-amino-acids : this is that the volume of gas obtained is greater, in varying degree, than is indicated by the equation :

$$H_3N^+{\cdot}CHR{\cdot}CO_2^- + HNO_2 \longrightarrow HO{\cdot}CHR{\cdot}CO_2H + N_2 + H_2O \quad . \quad . \quad . \quad . \quad (1)$$

It was in connexion with α-amino-acids that the special advantages of the procedure developed by Van Slyke (*J. Biol. Chem.*, 1911, **9**, 185; 1912, **12**, 275; 1929, **83**, 425) were most useful; but the uncertainty arising from the fact that "too much" nitrogen was evolved, together with a considerable amount of carbon dioxide, and that extensive frothing often occurred, made it apparent that the reaction was not adequately described by equation (1). Several of the amino-acids that gave anomalous gas volumes contained other NH_2-groups (amido) or peptide linkages (that could react after splitting); and, in the absence of analytical determination of the products, it was impossible to say that the excess of gas did not arise from this source. However, this uncertainty does not exist with glycine (and other monoamino-acids all the nitrogen in which is evolved), so that the excess of gas must have its origin connected in some way with the nitrite ion or nitrous acid. Van Slyke was of the opinion that the diazo-intermediate postulated could decompose in two ways, to give either the hydroxy-compound or, by total disruption of the molecule, carbon dioxide and "some other gas." Later investigators (Schmidt, *J. Biol. Chem.*, 1929, **82**, 587; Lough and Lewis, *ibid.*, 1934, **104**, 601; Kendrick and Hanke, *ibid.*, 1937, **117**, 161; 1940, **132**, 739; Viscontini, *Helv. Chim. Acta*, 1946, **29**, 1491) considered that oxidation by the nitrous acid was the source of the carbon dioxide, the nitrous acid being reduced to nitrogen. It has been suggested by others that nitrous acid de-amination may be a more complex reaction than is indicated by equation (1), and Treves and Salomone (*Biochem. Z.*, 1908, **7**, 11) and Dunn and Lewis (*J. Biol. Chem.*, 1921, **49**, 327) observed the red colour of the alkaline solution after the deaminising of certain proteins. In the absence of further data they were unable to suggest the nature of these secondary reactions. The present paper is confined to the general chemistry of the reaction of nitrous acid with glycine, and forms part of an investigation dealing with the mechanism and products of nitrous acid–amine reactions.

A clue to the more detailed behaviour of the nitrous acid reaction with α-amino-acids was obtained during a study of the reaction of methylamine with nitrous acid (to be published later). It was found that an appreciable amount of nitromethane could be isolated. It was postulated, by analogy, that under similar conditions the corresponding α-nitro-carboxylic acid may be formed in reaction (1); and it is well known that such compounds are readily decarboxylated to yield nitroparaffins. Moreover, such α-nitro-acids would be expected to be very reactive to nitrous acid, and it seemed plausible that this was the cause underlying the anomalous behaviour of the α-amino-acids and, in particular, the source of the carbon dioxide. Although the present investigation has been confined to the first member of the series, glycine, considerable support was given to the above postulate when it was found that the greater the nitrite-ion concentration in the reacting solution the greater was the excess of evolved gas over the amount predicted by equation (1). Furthermore, the reaction residue became red when made alkaline; this colour could be discharged on acidification, and ether-extraction from the acid solution yielded pale yellow needles of methyl nitrolic acid.

FIG. 1.



Evolution of gas from glycine (0·1 *mole*) + *sodium nitrite* (0·6 *mole*) *in water* (300 *ml.*) *when slowly acidified at* 16° *and then heated.*

The reaction was carried out by slow addition of acid to the glycine solution containing the appropriate amount of sodium nitrite. The nature of the gas evolution is shown in Fig. 1. A rapid evolution of gas consisting of nitrogen (~75%) and carbon dioxide (~25%) was followed by a slow, but steady, evolution of a mixture of nitrogen, carbon dioxide, and *nitrous oxide*, together with nitric oxide from decomposition of the nitrous acid. This slow secondary gas evolution was observed by Levene and Van Slyke (*J. Biol. Chem.*, 1912, **12**, 285) and Schmidt (*loc. cit.*), although its nature was not investigated. Initially the reaction solution is almost colourless, but, on the addition of acid, it quickly develops a reddish-brown colour, which soon becomes green. In order to accelerate the slow gas evolution the reaction products were heated after 2—3 hours. When heated, the solution became very dark green, and much gas was evolved. The solution then suddently became paler—to a reddish-orange, and finally to a very pale yellow—and the gas evolution ceased. The final volume of gas obtained is shown by the broken line in Fig. 1. A pronounced odour of hydrogen cyanide was always observed after this final heating.

With greater concentrations of sodium nitrite the phenomena were essentially the same, but the volume of gas obtained was considerably increased, particularly that of carbon dioxide. These results are shown in Table I.

Considerable frothing occurred during reaction at the two higher nitrite concentrations and it was with some difficulty that this was controlled at the highest concentration. This moderately stable froth, which tends to cause the reaction contents to pass over into the gas-collecting apparatus, is discussed below.

Table I.

Gas evolution (N.T.P.) *when* 80 *ml. of hydrochloric acid* (2·0N.) *are slowly added to glycine* (0·1 *mole*) *and sodium nitrite* (*x mole*) *in* 300 *ml. of water. Theoretical volume according to equation* (1), 2·24 *l.*

Amount of glycine, mole.	Initial amount of $NaNO_2 = x$, mole.	N_2, l.	CO_2, l.	N_2O, l.	$\frac{N_2\ (\text{expt.})}{N_2\ (\text{theor.})}$.	$\frac{[\text{Nitrite}]}{[\text{Glycine}]}$.
0·100	0·300	2·45	0·69	0·32	1·09	3
0·100	0·600	2·68	0·97	0·37	1·19	6
0·100	1·200	2·72	1·13	0·45	1·23	12

The amount of glycollic acid in the residues showed a progressive decrease, corresponding to the anomalous results described above; these results are contained in Table V.

When the reaction between glycine and nitrous acid was carried out in the cold with very slow generation of nitrous acid it was possible to isolate nitromethane by distillation and fractionation of the residue. If, however, the acid was added at all rapidly, nitromethane could not be isolated and ether-extraction of an unheated portion of the solution yielded pale yellow crystals of methylnitrolic acid (together with a yellow oil—see below). This behaviour was consistent with that of nitroacetic acid, as was directly shown by experiment. The successful isolation of the products of the self-decomposition of nitroacetic acid on the one hand, and also of the products of its reaction with nitrous acid on the other, confirms beyond reasonable doubt its formation in the above reaction. It has not, however, been possible to isolate nitroacetic acid from the reaction mixture, for the above reasons, and in order to check the hypothesis further and at the same time obtain an estimate of the amount of nitro-acid formed, the empirical method was adopted of running a solution of synthetically prepared nitroacetic acid into a solution of sodium nitrite which was being slowly acidified. In this way one would be imitating the abnormality postulated in the glycine reaction, and one would look for all the abnormal behaviour observed in this reaction, *viz.*, (1) excessive yield of nitrogen, (2) the formation of carbon dioxide and nitrous oxide, (3) the striking colour changes : colourless ⟶ reddish-brown ⟶ dark green, and, on heating, very dark green ⟶ reddish-orange ⟶ pale yellow, (4) the extensive frothing, (5) the strong odour of hydrocyanic acid on distillation and fraction of the reaction residues. The duplication of behaviour was exact. Table II shows the quantities of gas evolved when potassium nitroacetate reacts with nitrous acid and the resultant solution is heated.

Table II.

Potassium nitroacetate, g.	N_2, l.	CO_2, l.	N_2O, l.
4·00	0·22	0·58	0·26
6·00	0·26	0·80	0·30
Cf. the result of the reaction of glycine (0·1 mole) and sodium nitrite (0·3 mole) :			
	0·21 *	0·69	0·32

* Excess of nitrogen, unaccounted for on basis of equation (1).

This reaction of the nitroacetate ion with nitrous acid may be formulated as follows :

$$\mathrm{O_2N\cdot CH_2\cdot CO_2H + HNO_2 \longrightarrow [O_2N\cdot C(:N\cdot OH)\cdot CO_2H] \longrightarrow O_2N\cdot CH:N\cdot OH + CO_2} \quad . \quad (2)$$

The intermediate oximino-compound is immediately decarboxylated to give methylnitrolic acid, which then gives rise to nitrogen, carbon dioxide, and nitrous oxide, as shown below. The initial reaction in equation (2) probably involves the *aci*-form of the nitroacetate ion, as appears to be the case with other similar nitrosations, *e.g.*, the reaction of nitromethane with nitrous acid. When the reaction with nitroacetic acid was carried out at 0°, carbon dioxide was quantitatively evolved in accordance with equation (2), and ether-extraction of the solution yielded crystals of methylnitrolic acid together with the yellow oil—again in agreement with the behaviour with glycine. This oil is being further investigated; but it is thought to be dinitromethane, obtained from the oxidation of the methylnitrolic acid.

A reaction similar to that represented by equation (2) was observed by Ponzio (*Gazzetta*, 1903, **33**, I, 510). The action of dinitrogen tetroxide dissolved in ether on oximinoacetic acid caused decarboxylation, to give methylnitrolic acid. It would seem that the unstable intermediate postulated above is formed in this reaction too :

$$\mathrm{HO\cdot N:CH\cdot CO_2H \xrightarrow{N_2O_4} [HO\cdot N:C(NO_2)\cdot CO_2H] \longrightarrow HO\cdot N:CH\cdot NO_2 + CO_2}$$

Jovitschitsch (*Ber.*, 1895, **28**, 1213) isolated the ester of this unstable intermediate by the action of nitric acid (*d* 1·2) on ethyl oximinoacetoacetate.

Attention was next directed to the reactions of methylnitrolic acid, in order to determine the contributions of this substance to the reactions. In alkaline solution it has a red colour, which is destroyed on acidification. Wieland (*Ber.*, 1907, **40**, 418) has shown that this acid decomposes into fulminic acid and nitrous acid, and suggested the intermediate formation of the unstable isomeric nitrile oxide, $H{-}C{\equiv}N{\rightarrow}O$. By slow addition of alkali to an ice-cold solution of methylnitrolic acid he obtained a white precipitate which he formulated as a cyclic polymer of this isomer of fulminic acid and called trifulmin (*ibid.*, 1909, **42**, 808). If, however, the alkali is added to the solution at room temperature (20—25°) very extensive frothing occurs, and this has been shown to take place at pH 4—5. The above solutions of glycine and sodium nitrite, and of potassium nitroacetate and sodium nitrite are buffered approximately to this pH, and it is thus easy to understand the frothing. Wieland showed that in acid solution trifulmin was converted into formic acid and hydroxylamine hydrochloride, and, in alkaline solution, into cyanic acid; and he postulated the intermediate formation of formohydroxamic acid, $HO\cdot CH{:}N\cdot OH$, which he detected by colour tests with ferric ions (cherry-red) and cupric ions (green). Table III shows the quantities of gas evolved when a solution of synthetically prepared methylnitrolic acid was run into a solution of sodium nitrite that was being slowly acidified. The froth formed was vigorously stirred into solution. The striking colour changes noted above were again observed, and also the odour of hydrocyanic acid after the heating of the solution.

TABLE III.

Methylnitrolic acid, g.	N_2, l.	CO_2, l.	N_2O, l.
1·40	0·17	0·20	0·17
3·38	0·40	0·39	0·44

The dual mode of decomposition of formohydroxamic acid led to similar experiments being carried out on synthetically prepared acid, in order to determine the relative proportions of the two modes under the experimental conditions used, and also to ascertain the fate of the decomposition products in the nitrous acid solution. This decomposition of formohydroxamic acid may be represented :

$$NH_3 + CO_2 \longleftarrow HNCO \longleftarrow HO\cdot CH{:}N\cdot OH \longrightarrow H\cdot CO_2H + NH_2\cdot OH$$

The data in Table IV show the quantities of gas obtained when a solution of formohydroxamic acid is heated with an acid sodium nitrite solution (as in the experiments with glycine, with potassium nitroacetate, and with methylnitrolic acid).

TABLE IV.

Formhydroxamic acid, g.	N_2, l.	CO_2, l.	N_2O, l.
1·53	0·26	0·04	0·34

As expected, the cyanic acid formed in the reaction immediately decomposes to carbon dioxide and ammonia, the latter with nitrous acid then giving nitrogen. By a similar reaction part of the hydroxylamine gives nitrous oxide, and part decomposes to nitrogen and ammonia (which with nitrous acid gives nitrogen). The formic acid remains unchanged and may be detected by its reducing properties.

Most of the carbon dioxide obtained in the glycine reactions comes, therefore, from the decarboxylation of the nitrosated nitroacetic acid, and estimates were made on this basis, with the help of the data of Table II, of the amounts of nitro-acid formed in the different glycine–sodium nitrite solutions. These amounts are shown in Table V (col. 3), and compared with the analytical determinations of glycollic acid in the residues (col. 4).

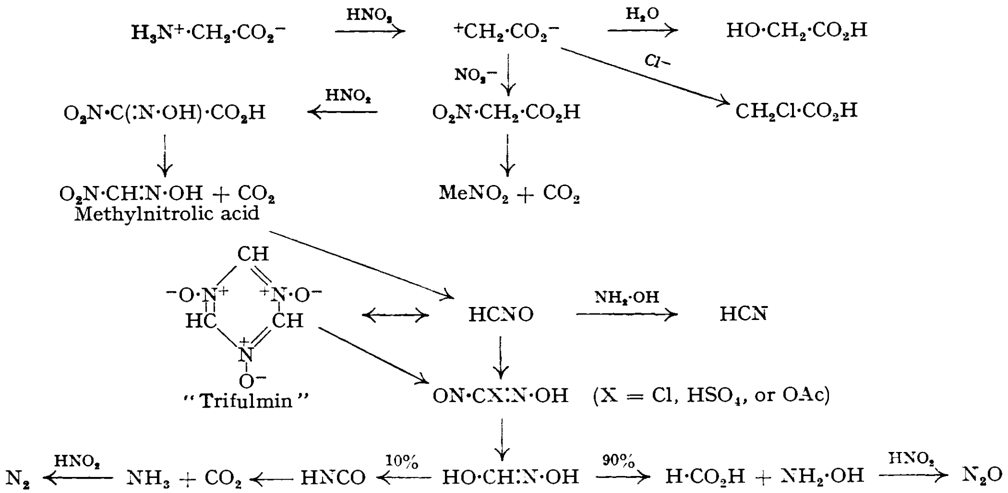
TABLE V.

Glycine, mole.	Initial, $NaNO_2$, mole.	Nitroacetic acid, mole.	Glycollic acid, mole.
0·100	0·300	0·03	0·055
0·100	0·600	0·04	0·039
0·100	1·200	0·05	0·032

The accumulated evidence now permits co-ordination of the behaviour observed when glycine reacts with nitrous acid in the presence of nitrite ion. The sequence of reactions may be formulated as shown in the reaction chart.

The detailed mechanism of the process involved in the removal of the NH_2-group has yet to be confirmed, but steps leading to a carbonium ion are postulated, and glycollic acid would be formed by interaction of this ion with water molecules. The carbonium ion may also react with negative ions in solution to give nitroacetic acid (which then undergoes the series of changes represented) or chloroacetic acid (which has been isolated), by interaction with nitrite and chloride ions, respectively. It would be expected that the greater the ionic concentration the greater would be the amount of substituted acid formed and, as shown in Table I, this has been found in the case of solutions containing varying amounts of nitrite ion.

In the reaction scheme the nitroacetic acid is represented as reacting with nitrous acid with *subsequent* decarboxylation, and not as undergoing preliminary decarboxylation followed by reaction of the nitromethane with the nitrous acid to give methylnitrolic acid. The reason



is that decarboxylation of nitroacetic acid is comparatively slow at 0° (and even at 20°), whereas in the presence of nitrous acid the carbon dioxide is liberated immediately. The nature of this reaction, which is used as a test for nitroacetic acid, does not appear to have been previously considered in detail.

The reaction solutions always developed a green colour, which was more intense the lower the temperature and the higher the nitrite-ion concentration. It was at first thought that methylnitrosolic acid ($ON{\cdot}CH{:}NOH$, from HONC and HNO_2) was the cause of the colour. This acid is known to be green and to decompose to formohydroxamic acid (Wieland and Hess, *Ber.*, 1909, **42**, 4175). However, methylnitrosolic acid is easily extracted by ether (to give a green extract), whereas the green colour in the glycine, nitroacetic acid, and methylnitrolic acid reaction solutions could not be removed in this way. Therefore, it appeared that the colour might be that of the anion of a more acidic nitroso-compound. The moderately stable green colour was obtained irrespective of the acid used (hydrochloric, sulphuric, acetic). When, however, hydrochloric acid was added in excess of that required for reaction the green colour became deep blue. This colour could easily be extracted by ether. On drying of the ethereal extract the colour changed to emerald-green. Evaporation of the ether under reduced pressure yielded a green oil, which was explosive at room temperature, and from its reactions was considered to be a chloronitroso-compound. Its constitution has yet to be confirmed. The green oil becomes blue when added to water, and, on addition of alkali, olive-green. These colour changes are reversible. This suggested that the green colour of the reaction solution might be caused by the ion of the chloronitroso-compound, $ON{\cdot}CCl{:}NO^-$ (which would be stabilised by resonance). With sulphuric and with acetic acid the anions formed would be $ON{\cdot}C(HSO_4){:}NO^-$ and $ON{\cdot}C(OAc){:}NO^-$, respectively. It is assumed that in these cases the free (un-ionised) acids are too unstable to exist in visible concentration. [Nef found that $CHCl{:}N{\cdot}OH$ is much more stable than $CH(HSO_4){:}N{\cdot}OH$; *Annalen*, 1894, **280**, 316.] The blue colour of the aqueous reaction solutions and the behaviour of the green oil were observed whenever hydrochloric acid was used in the treatment of glycine, nitroacetic acid, or methylnitrolic acid with nitrous acid. The gas evolutions were, however, independent of the acid employed.

The white solid obtained by Wieland (*loc. cit.*) on addition of alkali to methylnitrolic acid is very explosive when dry, and is insoluble in all ordinary solvents. Wieland considered it to be a trimer of an isomer of fulminic acid, and assigned to it a cyclic structure by analogy with the structures accepted at that time for cyanuric acid and cyamelide. In the light of present knowledge it seems that the properties of this substance would be more consistent with a straight-chain indefinite polymer $[\cdot CH{:}N^+(\cdot O^-)\cdot]_n$. The froth obtained in the experiments with glycine (cf. p. 150) appeared to be gas, from decomposition of part of the solid, enclosed in liquid films stabilised by the unchanged dispersed solid. As such it was difficult to suck dry on the filter funnel but, when dry, it had the same explosive properties as the more easily filtered substance obtained at 0° from methylnitrolic acid. One of the difficulties encountered in the Van Slyke determination of amino-acids by nitrous acid is the extensive frothing that often occurs, which tends to carry the reactants into the gas-measuring compartments. The cause of this was previously unknown, and octan-2-ol is added in these determinations in an attempt to diminish this trouble (Peters and Van Slyke, " Quantitative Clinical Methods," Vol. II, p. 385, Williams and Williams, Baltimore, Md., 1932). As shown above, the pH at which methylnitrolic acid changes to this polymer is about 4—5. The pH in a Van Slyke determination is in this range, and it is thus easy to see that any methylnitrolic acid formed during a glycine determination will lead to frothing.

The formation of hydrogen cyanide in all cases, starting from glycine, nitroacetic acid, or methylnitrolic acid, undoubtedly owes its origin to the oxidising properties of the unstable isomer of fulminic acid, and is correlated with the observation of Wieland (*loc. cit.*) that trifulmin and hydroxylamine gave hydrogen cyanide. Nef (*Annalen*, 1894, **280**, 330) and Schischkoff (*Annalen*, 1861, Suppl. Vol. **1**, 108) have also observed the formation of hydrogen cyanide from fulminic acid in reducing media.

It is clear that ion interaction is the determining factor causing the deviations from equation (1) ; and, in the usual procedure the anion involved is the nitrite ion. The subsequent changes that the nitroacetic acid thus formed may undergo allow an essentially complete explanation to be drawn up for the manifold series of changes observed in the glycine–nitrous acid reaction. The reaction is more disturbed by interaction with nitrite ion than by that with chloride ion ; for the latter, forming chloroacetic acid, may eventually yield the expected hydroxy-compound, whereas the nitroacetic acid, being so reactive to nitrous acid, results in extensive changes. Although it has generally been asserted that oxidation was the source of the carbon dioxide, it now appears safe to dismiss oxidation as the disturbing factor, for oxidation of amino-groups is not easy in acid solution and, moreover, the carbon dioxide is evolved from the commencement of the reaction. Also, very little nitric oxide ($<1\%$) is in the first part of the evolved gas. This gas contains some 25% of carbon dioxide and, if oxidation were the cause of this, much more nitric oxide would be expected. It was also shown by experiment that a large excess of nitrous acid was without effect on the amount of carbon dioxide, and that the deviation of behaviour from equation (1) depended on the nitrite ion and not on the nitrous acid.

In order to minimise this nitrite ion effect (and at the same time check again the above postulates) the procedure was adopted of running the nitrite solution slowly into an acid–glycine solution. The results obtained are summarised in Table VI. When the solution was acidified with hydrochloric acid the carbon dioxide was evolved in about 10% of its former quantity; but when acetic acid was employed, whilst less carbon dioxide was evolved, the quantity was considerably more than when hydrochloric acid was used. This was as expected; for the equilibrium

$$HOAc + NO_2^- \rightleftharpoons HNO_2 + OAc^-$$

lies very much to the left, and therefore much free nitrite ion is present in such a system.

TABLE VI.

Sodium nitrite solution slowly added to glycine (0·1 *mole*) *and* 0·12M-*acid in water* (300 *ml.*).

Acid.	N_2, l.	CO_2, l.
HCl	1·75	0·065
HCl	1·91	0·056
HOAc	2·24	0·234

The Van Slyke determination is carried out by mixing three solutions: saturated aqueous sodium nitrite, acetic acid, and the solution containing the amine. Acetic acid is used for two reasons. First, it causes less decomposition of nitrous acid—for the above-mentioned reason that very little free nitrous acid exists in these solutions. Nitrite ion is thus in excess, and so it causes the anomalous behaviour described. Secondly, amides, urea, etc., which are usually present in biological extracts, evolve nitrogen much less readily in the absence of mineral acid. Therefore attempts to minimise the disturbances caused by nitrite ion intervention by adding the nitrite solution to the amino-acid solution acidified with mineral acid (Table VI) would be unsatisfactory.

It was also noted that very little nitrous oxide was evolved when a glycine–sodium nitrite solution was rapidly acidified. This was due to the removal of nitrous acid from the solution (free nitrous acid rapidly decomposes). The hydroxylamine was shown to be held in solution as the hydrochloride.

From the foregoing, it is clear where the discrepancy lies in the Van Slyke determination of glycine. Although only one amino-acid has been studied here, it seems probable that the above series of reactions will apply in principle to a α-amino-acids generally, although the influence of adjacent groups in the higher members has yet to be ascertained. This influence may manifest itself with the higher amino-acids, such as alanine, etc., mainly by virtue of the reduction in the number of α-hydrogen atoms. The changes subsequent to the formation of nitro-compound may be impeded and nitrosation, which is so important in the rapid decarboxylation of glycine (p. 153), may not be possible. The amount of gas evolved with alanine, etc., is very close to that predicted by equation (1) and so permits the use of the Van Slyke determination, but it is almost certain that corresponding nitro-compounds are formed to a large extent and so the chemistry of the change is not represented by this equation. Glycine has always been recognised as one of the amino-acids least suitably determined by this method, and many attempts have been made to remove the anomaly of " too much " nitrogen. Kendrick and Hanke (*J. Biol. Chem.*, 1937, **117**, 161) claimed that the addition of potassium iodide or iodine eliminated the excess of nitrogen. This was disputed by Dunn and Porush (*ibid.*, 1939, **127**, 261) but reiterated by Kendrick and Hanke (*ibid.*, 1940, **132**, 739). They considered that the effect was caused by preferential oxidation by the iodine (with the result that nitrous acid was not reduced to nitrogen); but in view of the established nature of the glycine reaction it would be better to defer an explanation of the iodine effect until the reaction of iodine with the now known intermediates has been established.

The present investigation gives an explanation of the abnormal kinetic results of Abel, Schmid, and Sidon (*Z. Elektrochem.*, 1933, **39**, 863) in their investigation of glycine; it was found that as the nitrite-ion concentration was increased the velocity coefficient of the reaction increased several-fold. This was attributed to specific electrolyte effect on the amphoteric glycine (carbon dioxide was not tested for in the evolved gas, all of which was assumed to be nitrogen).

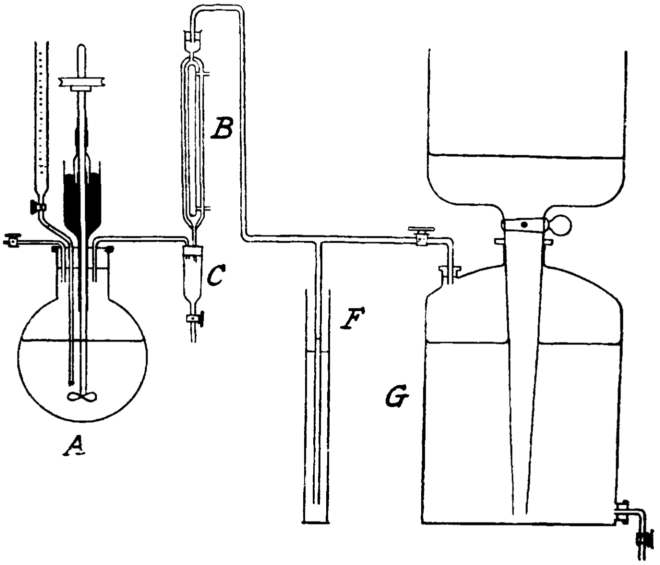
Viscontini (*Helv. Chim. Acta*, 1946, **29**, 1491) found that reaction of nitrous acid with polyglycines, such as glycylglycine, yielded very little of the expected hydroxy-compound, $HO{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CO_2H$, but a large amount of oxalylglycine, $HO_2C{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CO_2H$. The evolved gas contained very little carbon dioxide, but the nitrogen was 160% of that expected from the usual equation. These facts may now be interpreted on the assumption that the corresponding nitro-compound is formed, followed by nitrosation, giving $O_2N{\cdot}C(:N{\cdot}OH){\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CO_2H$ which decomposes to oxalylglycine. It seems probable that nitrous oxide formed part of the gas evolved, but this was not tested.

It may be asked : " Where does the sequence of reactions stop under the conditions of the Van Slyke determination of glycine ? " No proper answer can be given, but the considerable frothing that occurs indicates that part, at least, of the nitrolic acid has reached and passed the trifulmin stage. Concerning the gas evolution it may be said that there is a very rapid primary evolution of nitrogen (nitrous acid elimination of the amino-group of the amino-acid) accompanied by a fast evolution of carbon dioxide (from decarboxylation of the nitrosated nitro-acid). The secondary nitrogen and carbon dioxide, as well as the nitrous oxide, are formed at the end of the series of reactions (p. 153) and, at ordinary temperatures, this gas evolution is comparatively slow. The specified time in a Van Slyke determination is about 4 minutes (according to the temperature), and it can only be said that the stage reached in the gas evolution will at this time be somewhere just after the steep part of the curve in Fig. 1.

EXPERIMENTAL.

The apparatus used for measuring the gas evolution is shown in Fig. 2. Glycine (0·1 mole) and the appropriate amount of sodium nitrite were dissolved in water (300 ml.) in the flask *A* immersed in the thermostat. The whole apparatus was filled with nitrogen from a cylinder, and then standard acid (2N.) was slowly added from the burette (time of addition approx. 40 minutes). The evolved gas was collected in *G* over saturated aqueous sodium chloride, which was also saturated with the relevant gases. After a fixed interval of time (2—3 hours in different experiments) the thermostat was removed and the contents of flask *A* were heated to boiling. The ice-coldwater-condenser *B* re tained steam, and this condensate was collected in the container *C*. Hydrochloric, sulphuric, and acetic acid were used and no difference was observed (other than the blue colour when hydrochloric acid was used). The preliminary reaction (*i.e.*, before the heating) was carried out at 0°, 16°, or 25°, again with no significant difference (although the green colour that developed was more intense the lower the temperature) and 16° was chosen for convenience in the final investigation.

FIG. 2.



The gas analyses were carried out on samples (35—45 ml.) taken from the gas collectors and brought into contact successively with small volumes of concentrated absorbents in gas-burettes. Acid ferrous sulphate was used to remove nitric oxide, potassium hydroxide for carbon dioxide, and ethyl alcohol for nitrous oxide. The residual gas was assumed to be nitrogen. Tests for carbon monoxide were negative. Because of its solubility in most reagents the presence of nitrous oxide makes the analyses somewhat uncertain, but by the use of small volumes of concentrated reagents the error due to this was kept low. That the gas removed by the ethyl alcohol was nitrous oxide was proved by passing the original gas (after removal of nitric oxide and carbon dioxide) through a trap immersed in liquid air. A white solid was retained. On removal of the trap from the liquid air this white solid melted to a colourless liquid which boiled at a very low temperature, and the evolved gas remained colourless in the presence of oxygen. Samples were evaporated into a eudiometer, mixed with hydrogen (1·8—2·5 volumes), and exploded according to the method of Menzel and Kretzschmar (*Z. angew. Chem.*, 1929, **42**, 148). The contractions observed were exactly equal to the original volume of gas sample: $N_2O + H_2 \longrightarrow H_2O + N_2$.

Materials.—Glycine used in the above experiments was recrystallised from aqueous alcohol and contained no detectable impurities. Analysis by the Sörensen formaldehyde method gave results identical with 3 other samples of AnalaR glycine.

Potassium nitroacetate was prepared from nitromethane (Steinkopf and Kirchhoff, *Ber.*, 1909, **42**, 3925) and, when recrystallised several times (50% aqueous potassium hydroxide) and washed with ethyl alcohol, was pale yellow. It was quite stable when kept *in vacuo*. It proved more convenient to use the potassium salt rather than the free acid, the preparation of which required the extra steps given by Pedersen (*Trans. Faraday Soc.*, 1927, **23**, 316). The acid, after recrystallisation from chloroform, melted at 93—93·5° (lit., 87—89°).

Methylnitrolic acid was prepared from nitromethane by the method of Wieland (*loc. cit.*). It is an unstable compound, m. p. 64—66° (decomp.), and could not be kept for more than a few hours. The aqueous solution soon decomposed and the gas measurements obtained with this (Table III) are not as decisive as those obtained using potassium nitroacetate.

Formohydroxamic acid, prepared from ethyl formate and hydroxylamine (Jones, *Amer. Chem. J.*, 1898, **20**, 28), formed white glistening plates, m. p. 80°.

Isolation of Nitromethane from the Glycine–Nitrous Acid Reaction.—The success of this experiment depends on the very slow addition of acid to the glycine–sodium nitrite solution in order to avoid a local excess of nitrous acid and so to enable the nitroacetic acid to decompose to nitromethane. Glycine (0·5 mole) and sodium nitrite (4 mole) in water (500 ml.) were slowly acidified (150 ml. of 2N-sulphuric acid) during 3 hours. An hour after completion of the acidification the solution was distilled. Oily droplets were visible in the early portions of the distillate, and distillation was continued until 150 ml. were collected. The distillate was neutralised and then fractionated using an ordinary disc-column. Between 75° and 95° a colourless 2-phase liquid was collected (total volume, 10 ml.). The lower phase (~2·5 ml.) was separated. With nitrous acid it gave the nitrolic acid test for nitromethane and, after being dried ($MgSO_4$), had b. p. 100—102° (micro-method) (nitromethane has b. p. 101°) (Found: C, 20·1; H, 5·5; N, 22·4. Calc. for CH_3O_2N: C, 19·7; H, 4·9; N, 22·9%). The refractive index was identical with that of a carefully purified specimen of nitromethane.

Isolation of Methylnitrolic Acid from the Glycine–Nitrous Acid Reaction.—The reaction was carried out at 0° with moderately quick addition of acid, taking precautions to keep the temperature low. The solution was extracted twice with ether, the extract dried ($CaCl_2$), and the solvent removed under reduced pressure. Pale yellow needles (~2 g.), m. p. 64—66°, were obtained from a solution of glycine (0·4 mole) and sodium nitrite (1·2 moles) in 300 ml. of water when acidified by 300 ml. of 2N-sulphuric acid; the m. p. and reactions were identical with those of synthetically prepared methylnitrolic acid. About 5—10 ml. of a yellow oil were also obtained in the extraction (cf. p. 151). On increase of the concentration of sodium nitrite in the above experiment, there was a marked increase in the amounts of yellow solid and oil, but these experiments at higher nitrite concentrations were difficult to control because of the extensive frothing.

Identical behaviour was observed when potassium nitroacetate was used in place of glycine. Pale yellow needles, m. p. 64—66° (decomp.), and a yellow oil were obtained on ether-extraction of the reaction residue.

Formation of Trifulmin.—The white froth that formed at ordinary temperatures in both the glycine and the potassium nitroacetate experiments could be filtered off; it had the same explosive character as the substance prepared from methylnitrolic acid (Wieland, *loc. cit.*). With methylnitrolic acid the frothing was shown to commence at pH 4—5.

Isolation of Chloroacetic Acid from the Glycine–Nitrous Acid Reaction when Chloride Ions were present.—Glycine (0·3 mole), sodium nitrite (0·32 mole) and potassium chloride (2·0 moles) were dissolved in water (500 ml.), and 150 ml. of acid (2N.) were slowly added during 2 hours. The temperature was kept at 5°. The blue colour of the final solution was readily removed with ether, and the colourless solution was then extracted with several further lots of ether. These extracts were dried ($CaCl_2$). Evaporation of the ether yielded 11·9 g. of white crystalline material. This had an odour similar to that of acetic acid, contained "bound" chlorine, and, after recrystallisation from chloroform, had m. p. 63°, undepressed on admixture with chloroacetic acid. The material boiled without decomposition at 187—189°/765 mm. (Found: C, 25·6; H, 3·3; Cl, 37·4. Calc. for CH_3O_2Cl: C, 25·4; H, 3·2; Cl, 37·6%). The amount of recovered chloroacetic acid represents a 40% conversion of the original glycine.

Analytical Determination of Glycollic Acid in the Glycine–Nitrous Acid Residues.—The acid–alkaline permanganate method suggested by Wieland and Franke (*Annalen*, 1927, **457**, 13) with the recommendations of Bottger and Oesper ("Newer Methods of Volumetric Chemical Analysis," Van Nostrand, N.Y., 1938) was found satisfactory. It was shown, using potassium nitroacetate, that most of the products other than glycollic acid could be removed by treatment with excess of nitrous acid followed by boiling (to remove formic acid). It was shown, using glycollic acid (Kahlbaum), that the above treatment resulted in the decomposition of approx. 10% of the glycollic acid, but it was considered that the method was sufficiently accurate for the present purpose.

Other Identifications.—Hydrocyanic acid was detected in the distillates by the characteristic odour, and the white precipitate obtained with silver nitrate (soluble in ammonia, insoluble in nitric acid) darkened on addition of mercurous nitrate (dissolving in excess). Prussian blue was obtained by heating with ferrous sulphate in alkaline solution. The pungent odour obtained on continued distillation (and fractionation) was established as due to formic acid by the solubility of the silver salt of the latter, and its reduction of ammoniacal silver nitrate. Hydroxylamine was detected in the residues by its reducing properties (mercuric chloride), and the formation of ammonia on heating of the alkaline solution.

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