702. *The Reaction of Nitrous Acid with Glutamine and Glutamic Acid.*

By A. T. Austin and J. Howard.

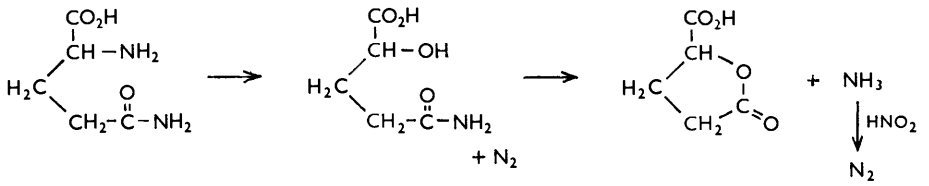
The anomalous reaction of glutamine with weakly acidic solutions of nitrous acid (Van Slyke determination), where 100% of the total nitrogen is evolved instead of the " expected " 50%, occurs because the carbonium ion, which is formed by the action of nitrous acid on the α-amino-group, undergoes intramolecular cyclisation with the oxygen of the amido-group. The hydroxy-amino-compound subsequently formed then reacts with nitrous acid, giving a lactone and nitrogen. If the concentration of nitrous acid is low the hydroxy-amino-compound decomposes to a lactone and ammonia, and the gas evolved falls to half the volume obtained under ordinary Van Slyke conditions. Glutamic acid also forms a lactone when treated with nitrous acid.

The reaction of glutamine (α-aminoglutaric acid γ-amide) with weakly acidic solutions of nitrous acid is anomalous in that 100% of the nitrogen is evolved instead of 50%. Under conditions of low acidity amido-groups are usually inert towards nitrous acid (undergoing less than 1% reaction in 30 min.) and the reason for the reactivity of this group in glutamine has caused much speculation. Glutamine stands in contrast to its lower homologue, asparagine (α-aminosuccinic acid β-amide), whose behaviour towards weakly acidic solutions of nitrous acid is " normal " in that only the α-amino-group reacts and 50% of the nitrogen is evolved. This unusual behaviour of glutamine was first reported by Schulze and Bosshard [1] in 1883 and re-discovered by Chibnall and Westall [2] in 1932. The latter workers accepted an explanation put forward by Plimmer [3] to account for the catalytic effect of mineral acids on the acid amide–nitrous acid reaction * and stated

* This was that amides normally existed in the imido-form, NH:CR·OH, which in the presence of mineral acid became converted into the amido-form, R·CO·NH_2. Only the latter, containing a primary NH_2-group, was considered capable of undergoing reaction with nitrous acid to liberate nitrogen.

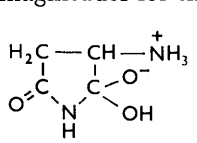
[1] Schulze and Bosshard, *Landw. Versuchs-Stat.*, 1883, **29**, 295.
[2] Chibnall and Westall, *Biochem. J.*, 1932, **26**, 122.
[3] Plimmer, *J.*, 1925, **127**, 2651.

that as the amide group in glutamine reacts readily with nitrous acid in the presence of merely acetic acid it must possess the amido-structure $R{\cdot}CO{\cdot}NH_2$ in the absence of mineral acid and in this way be different from other amides. Lichtenstein,[4] however, showed that γ-*N*-methyl- and γ-*N*-ethyl-glutamine liberated 90% and 88%, respectively, of their nitrogen in the Van Slyke determination and this was clearly inconsistent with the conclusion that only amides possessing the $R{\cdot}CO{\cdot}NH_2$ structure could react with nitrous acid to yield nitrogen. Lichtenstein suggested that the reaction of nitrous acid with glutamine and the substituted glutamines involved the replacement of the α-amino-group by hydroxyl to give the α-hydroxy-γ-amide with evolution of one mol. of nitrogen. The hydroxy-amide then underwent lactonisation with liberation of ammonia (or the alkylamine) which gave the second mol. of nitrogen on reaction with the nitrous acid:



Archibald [5] showed this suggestion to be unacceptable on the grounds that free ammonia reacts to the extent of only 25% in 5 minutes in the Van Slyke determination whereas Lichtenstein's results required that 80% reaction should have taken place.

A shift of ground then focused attention on the amide group of asparagine. It was suggested [6] that this group was less reactive than the amide group in glutamine by postulating that asparagine existed in aqueous solution mainly in a cyclic form in which the nitrogen of the amide group formed part of the ring (see A). Leach and Lindley,[7] however, pointed out that such cyclic formulations would represent asparagine and isoasparagine as substances capable of interconversion in solution whereas this interconversion has not been observed. They also cited as evidence against the postulated cyclic form the fact that the structure of glycylasparagine, as determined by *X*-ray analysis, showed the amide and the carboxyl group of the asparagine part of the molecule to be disposed *trans* to each other.[8] The similarity of kinetic order and magnitudes for the rate of hydrolysis of the amide group in asparagine and in asparaginyl-glycine (where no ring formation was considered possible) led Leach and Lindley [9] to contend that the amido-group in each of these molecules is of the same type and is not part of a cyclic structure. This contention received support when the structure determination of asparagine hydrate [10] showed the carboxyl and the amido-group to be disposed *trans* to each other, thus ruling out the possibility of a cyclic structure in the solid. The same open-chain structure has also been established for glutamine,[11] and the bond distances and bond angles of the amide group in this molecule were shown to be very similar to those in acetamide.



(A)

The failure to reveal any fundamental difference between the structure of amide groups in glutamine, asparagine, and acetamide and the lack of experimental evidence to support the reaction steps proposed by Lichtenstein made it desirable to re-examine this problem, for it was felt that the principle concerned in the anomalous behaviour of glutamine might

[4] Lichtenstein, *J. Amer. Chem. Soc.*, 1942, **64**, 1021.
[5] Archibald, *Chem. Rev.*, 1945, **37**, 161.
[6] Steward and Thompson, *Nature*, 1952, **169**, 739.
[7] Leach and Lindley, *Nature*, 1953, **171**, 1062.
[8] Katz, Pasternak, and Corey, *Nature*, 1952, **170**, 1066.
[9] Leach and Lindley, *Trans. Faraday Soc.*, 1953, **49**, 915.
[10] Saito, Cano-Corona, and Pepinsky, *Science*, 1955, **121**, 435.
[11] Cochran and Penfold, *Acta Cryst.*, 1952, **5**, 644.

be of wider application in organic chemistry. Ground-state structures are often of no more than ancillary help in elucidating the nature of chemical reactions and it seemed that a kinetic investigation constituted the best approach. Sachs and Brand [12] have shown qualitatively that a lactone is formed in the deamination of glutamine (and they postulated that this was formed by cyclisation of the initially formed carbonium ion) but in the absence of kinetic data their observations apply to other mechanisms that can be formulated for the reaction. The present investigation was designed to establish the kinetic form for this reaction and, by measuring the reactivity of the only plausible intermediate, it has been possible to put forward a mechanism that accounts for the reactivity of the amide group in glutamine.

Results

Formation of Lactone.—The formation of a lactone in the deamination of glutamine by nitrous acid was shown qualitatively by using the hydroxamic acid test, and the identity of the lactone was established as γ-carboxy-γ-butyrolactone by comparison of the infrared

Fig. 1. *Reaction–time curve for deamination of glutamine and the appearance of lactone in aqueous nitrous acid solution.* 0·02M-*Glutamine* + 0·1M-*nitrous acid at* 24·6° *and* pH 2·4.

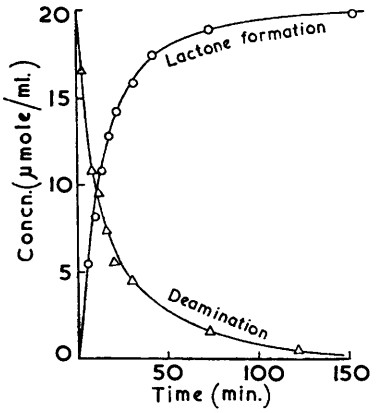
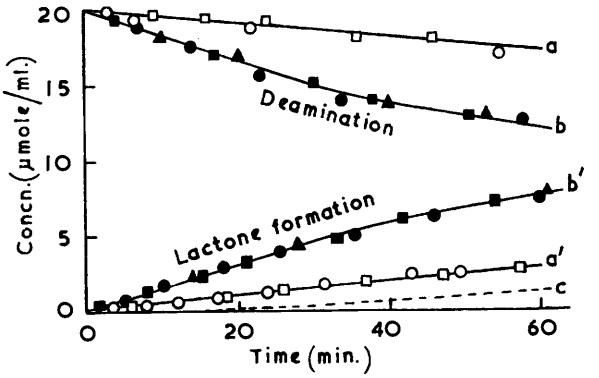


Fig. 2. *Effect of nitrous acid concentration on the rate of deamination* (a, b) *and of lactone formation* (a′, b′) *at* 24·6° *and* pH 2·6, P_{NO} = 1 atm.



Initial concns.: (a) [Glutamine] = 0·02, [Nitrous Acid] = 0·02M. (b) [Glutamine] = 0·02, [Nitrous Acid] = 0·04M.

spectrum of the isolated compound with that of an authentic specimen of this lactone. Deamination of glutamic acid was likewise shown to produce the same lactone. Treatment of γ-*N*-methylglutamine with nitrous acid also resulted in a lactone, but no lactone was detected when aspartic acid was treated with nitrous acid under the same conditions.

Rate of Deamination of Glutamine and Rate of Formation of Lactone.—The two curves in Fig. 1 show, severally, the rate of disappearance of glutamine from aqueous nitrous acid solutions of this compound and the rate of formation of lactone in the same solution. They show that the disappearance of the amino-compound occurs at approximately the same rate as the appearance of the lactone over the whole course of the reaction.

Free ammonia was formed in solution during these runs and the ninhydrin colour determinations of the unchanged glutamine had to be corrected to allow for the proportion of colour produced by the reaction of ninhydrin with the ammonia (see p. 3599 for a discussion of the formation of ammonia in these runs). When glutamic acid was treated with nitrous acid in the above manner a similar pair of curves was obtained, showing that the deamination of glutamic acid resulted in the simultaneous formation of γ-carboxy-γ-butyrolactone. In this case no ammonia is formed and no correction to the ninhydrin readings was necessary.

A series of reaction–time measurements for the disappearance of amino-compound and the appearance of lactone was carried out in which the amino-compound was determined by the copper complex method of Pope and Stevens (p. 3602). This determination is unaffected by the

[12] Sachs and Brand, *J. Amer. Chem. Soc.*, 1954, **76**, 3601.

presence of free ammonia. Fig. 2 shows the results of two sets of experiments carried out at different concentrations of nitrous acid.

By using the method of initial rates it can be seen from the gradients of a and a', and b and b', in Fig. 2, that the lactone is formed in both solutions at 97% of the rate of the deamination. Curve c in Fig. 2 shows the calculated amount of lactone (× 1000) that would have been formed by lactonisation of α-hydroxyglutaric acid (from the measured rate of lactonisation of the hydroxy-acid) and it can be seen that the amount of lactone formed after 80 min. in the glutamine–nitrous acid reaction is approximately 4500 times the amount that could have been formed by lactonisation of the α-hydroxy-acid (see below the reasons for using this acid for comparison).

Kinetic Order for the Reaction.—Fig. 2, curve a, and Fig. 3 show that doubling the initial concentration of glutamine caused the reaction to go twice as fast and Fig. 2, curves a and b,

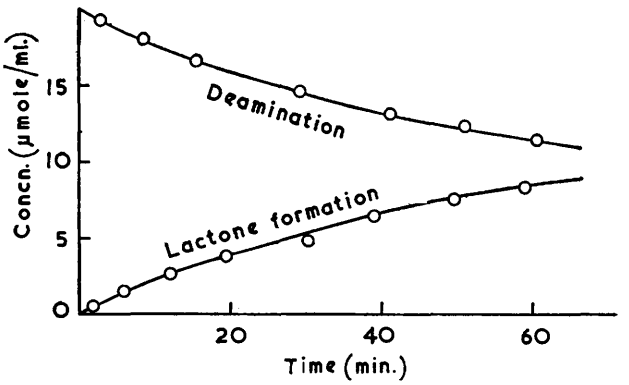


FIG. 3. *Reaction–time curve for* [Glutamine] = 0·01 + [Nitrous Acid] = 0·04M *in aqueous solution at* 24·6° *and* pH 2·6, p_{NO} = 1 atm.

that the doubling of the initial concentration of nitrous acid increased the initial rate four-fold. The overall rate expression for the glutamine–nitrous acid reaction is therefore:

$$v = k[\text{Glutamine}][\text{Nitrous acid}]^2$$

where k = 13·3 ± 1·3 mol.$^{-2}$ l.^{2} min.$^{-1}$ at 24·6°.

Amount of Lactone Formed Relative to the Amount of Glutamine Deaminated.—The percentage of deamination of glutamine equivalent to lactone formation given earlier was derived by comparison of the respective initial rates. However, as the amount of lactone formed from α-hydroxyglutaric acid is extremely small, it is possible to obtain a more accurate estimate of the percentage of deamination resulting in lactone formation by comparing directly the amounts of glutamine deaminated with the amount of lactone found at any one time. On averaging readings, taken at 10 min. intervals, from the curves in Fig. 2 and 3 the value 93 ± 3% is obtained for the percentage of deamination leading to lactone formation. This figure is accurate to within 3%, whereas the value obtained from the initial rates (96—97%) is limited to the 5% accuracy that was possible in measuring the gradients.

Rate of Deamination of Glutamic Acid and Rate of Formation of Lactone.—Fig. 4 shows the reaction–time curves for the disappearance of glutamic acid from aqueous nitrous acid solutions of this compound and the rate of formation of lactone in the same solution. The similarity of these two curves with the corresponding ones for glutamine (Fig. 2, curves a and b) indicates that the same processes occur with both the amino-compounds in question and that the lactone is formed in a reaction that is fast by comparison with the rate of lactonisation of the α-hydroxyglutaric acid (curve c).

The identical behaviour for these two items (deamination/lactone formation and the ratios thereof) for glutamine and glutamic acid meant that the γ-carboxamide group and the γ-carboxyl group must react with the carbonium ion at similar (fast) rates. We accordingly felt justified in using the measured rate of lactonisation of the α-hydroxy-acid only, to compare possible origins of lactone in the reactions of both glutamine and glutamic acid. In the absence of this identical behaviour we should have felt obliged to measure the rate of lactonisation of the α-hydroxy-γ-amide.

Nature of the Gas Evolution.—The formation of ammonia during the kinetic experiments was unexpected. Chibnall and Westall had observed that glutamine liberated 92% of the

total nitrogen in the Van Slyke determination; this ruled out the possibility that ammonia was formed under the conditions of this determination, for it is known (and our experiments confirmed it) that ammonia undergoes only partial (36%) reaction in the Van Slyke determination. Experiment further showed that glutamine yielded 93% lactone in the time prescribed for this determination. This observation, together with the kinetic finding that the lactone is formed at a rate very similar to that of deamination, indicated that the conditions of the kinetic runs and the Van Slyke determination were sufficiently different to permit different reactions to occur after the cyclisation (a necessary preliminary for lactone formation) had taken place. The most obvious difference appeared to be the respective concentrations of

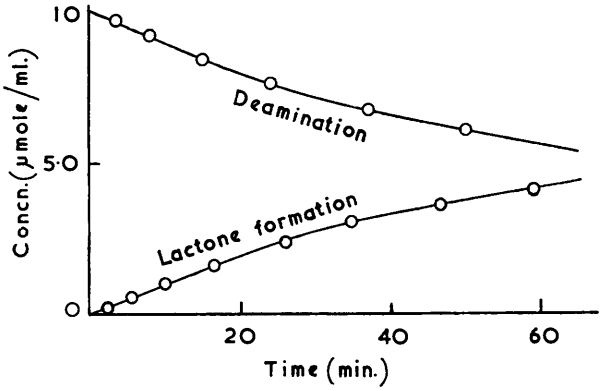


FIG. 4. *Reaction–time curves for* [Glutamic Acid] = 0·02M + [Nitrous Acid] = 0·04M *in aqueous solution at* 24·6° *and* pH = 2·7, p_{NO} = 1 atm.

nitrous acid. Table 1 shows the volumes of gas obtained in the Van Slyke determinations when the concentration of nitrous acid was progressively lowered.

These results show that the higher the concentration of nitrous acid and sodium nitrite the greater is the volume of nitrogen liberated. It will be shown below (p. 3601) that intervention of the nitrite ion can be ruled out and the above results must be interpreted in terms of the effect of nitrous acid concentration alone.

The volume of gas obtained was measured with approximately the same concentrations of

TABLE 1. *Gas volumes obtained in Van Slyke determination of glutamine with varying* [HNO_2 + $NaNO_2$] *at* pH = 3·7.

Initial [HNO_2 + $NaNO_2$] *	Reaction time (min.)	Total nitrogen (%)	Initial [HNO_2 + $NaNO_2$] *	Reaction time (min.)	Total nitrogen (%)	Initial [HNO_2 + $NaNO_2$] *	Reaction time (min.)	Total nitrogen (%)
0·86M	8	98·3	0·54M	8	87·4	0·27M	8	69·3
	30	100·0		10	88·0		30	85·0
	165	100·0		34	90·3			
				180	93·5			

* The reagents used in the Van Slyke determination generate *in situ* nitrous acid, quite dilute (0·05M) solutions of which readily decompose. It is thus only permissible to refer to initial concentrations of nitrous acid and sodium nitrite.

nitrous acid as were used in the kinetic runs; and the pH was varied over the range 2·3—3·3. In all determinations, irrespective of whether sulphuric acid or acetic acid was employed, only 50% of the total nitrogen was evolved.

Gas evolution took place at approximately the same rate as during formation of lactone in the kinetic runs. After the 50% gas evolution 2—3% more gas was liberated in the next 13 hours. This showed that the ammonia in solution was not attacked to any significant extent under these conditions, and any disturbance from this source could be ignored in the kinetic experiments.

Composition of Evolved Gas in the Van Slyke Determination of Glutamine and Related Compounds.—The gas absorbent used in the normal Van Slyke procedure (alkaline potassium permanganate) prevents the detection of any carbon dioxide or nitrous oxide in the evolved gas; accordingly the gas evolved was treated in turn with selective absorbents, *viz.*, acid ferrous sulphate (NO); sodium hydroxide (CO_2); ethyl alcohol (N_2O). The residual gas was assumed to be nitrogen, after negative tests for carbon monoxide with ammoniacal cuprous chloride.

The composition of the gases evolved in the Van Slyke determination of glutamine and of alanine are shown in Table 2, together with the gases (if any) evolved when the deaminated residues were heated.

TABLE 2. *Composition of gas evolved in the Van Slyke determination of glutamine and alanine at 20° and after heating to 100°.*

		Gas volume as % of total nitrogen in amino-acid			
Amino-acid	Temperature	N_2	CO_2	N_2O	CO
Glutamine	20°	98	Nil	Nil	Nil
,,	After heating rection residues	Nil	Nil	Nil	Nil
Alanine	20°	100	35	4	Nil
,,	After heating reaction residues	50	10	Nil	Nil

These results show that pure nitrogen is evolved in the deamination of glutamine, whereas the gas obtained from alanine indicates considerable deviation from simple deamination.

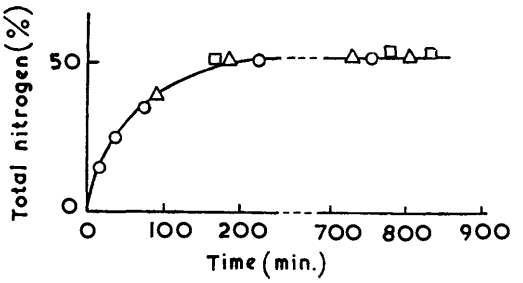


FIG. 5. *Percentage of total nitrogen obtained in modified Van Slyke determination of glutamine.*
[Glutamine] = 0·01M, [HNO_2] = 0·06M.

γ-*N*-Methylglutamine gave 86% of lactone in the Van Slyke determination and 85% of the total nitrogen was evolved. If the determination was slightly modified so that, relative to the amino-compound, a higher concentration of nitrous acid was present, 95% of the total nitrogen could be obtained. There was also about 2% of carbon dioxide in the evolved gas.

It was of interest to ascertain the behaviour of isoglutamine in the Van Slyke determination as in this case the cyclisation envisaged for the glutamine–nitrous acid reaction could not occur and only 50% of the total nitrogen should be liberated. Table 3 shows that, while only slightly

TABLE 3. *Composition of gas evolved in the Van Slyke determination of isoglutamine at 20° and after heating to 100°.*

	Gas volume as % of total nitrogen in amino-acid		
Temperature	N_2	CO_2	N_2O
20°	55	5	2
After heating reaction residue to 100°	25	6	5

more than 50% of the total nitrogen is evolved at 20°, the presence of carbon dioxide and nitrous oxide in the collected gas indicates considerable deviation from simple deamination. The deaminated solution, at 20°, gave a faint red colour when alkaline, indicating the presence of nitrolic acids.

Comparative experiments with α-alanine amide showed its behaviour to be similar to that of isoglutamine and 55% of the total nitrogen was evolved in the Van Slyke determination at room temperature; similar measurements of the gas evolved from propionamide showed that only 2% of reaction occurred in 15 minutes at 70°.

DISCUSSION

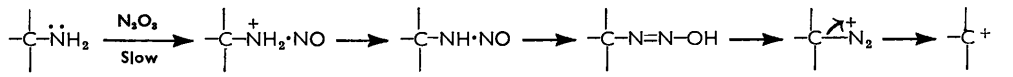
The rate expression for the nitrous acid deamination of glutamine is of the form:

$$v \propto [\text{Amine}][\text{Nitrous acid}]^2$$

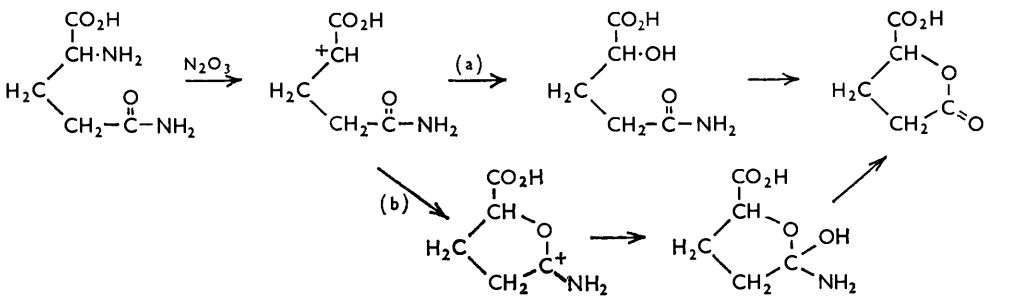
and this is the same as the expression originally obtained for a series of amines and amino-acids by T. W. J. Taylor [13] in 1928. Following Hammett,[14] Hughes and Ingold's school,[15]

[13] Taylor, *J.*, 1928, 1099, 1897; Taylor and Price, *J.*, 1929, 2052.
[14] Hammett, " Physical Organic Chemistry," McGraw-Hill Book Co., New York, 1940, p. 294.
[15] Brewster, Hiron, Hughes, Ingold, and Rao, *Nature*, 1950, **166**, 179.

and Whitmore [16] the reaction is considered to involve a rate-determining *N*-nitrosation by interaction of N_2O_3 ($\propto [HNO_2]^2$) with the free amino-group, leading, by subsequent fast steps, to a carbonium ion:

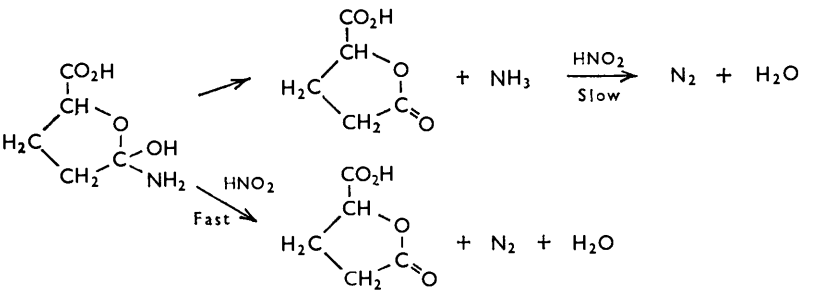


The carbonium ion, $^+CH(CO_2H)\cdot[CH_2]_2\cdot CO\cdot NH_2$, formed in the nitrous acid deamination of glutamine could lead to the product, γ-carboxy-γ-butyrolactone, by two processes: (*a*) by lactonisation of the hydroxy-amide formed by interaction of the carbonium ion with water and (*b*) by intramolecular interaction between the electron-deficient centre of the carbonium ion and the oxygen of the amide group to form a cyclic carbonium ion which with water gives a hydroxy-amino-compound that in turn rapidly gives the lactone:



As the lactone appears in the reaction at 93% of the rate of deamination of the amine the processes involved in the formation of lactone from the carbonium ion must be fast and, since the α-hydroxyglutaric acid undergoes lactonisation only very slowly at the pH of the deamination, the route to the lactone through the hydroxy-acid has been rejected. Route (b) must then be considered.

Liberation of ammonia during the kinetic runs first showed that the postulated hydroxy-amino-compound must be unstable under faintly acid conditions, and experiment showed that it was able to behave in two ways which are determined by the concentration of nitrous acid. If this was small, only 50% of the total nitrogen in glutamine was liberated quickly and this was followed by a very slow evolution of gas (Fig. 5). On the other hand, if the concentration of nitrous acid was high (Van Slyke conditions) approximately 100% of the nitrogen was evolved in 4 minutes. These observations can be explained by assuming that in the absence of an appreciable concentration of nitrous acid the hydroxy-amino-compound undergoes simple hydrolysis to the lactone and ammonia (which reacts very slowly with nitrous acid), whereas in the presence of much nitrous acid it reacts rapidly with this reagent to form the lactone and nitrogen:

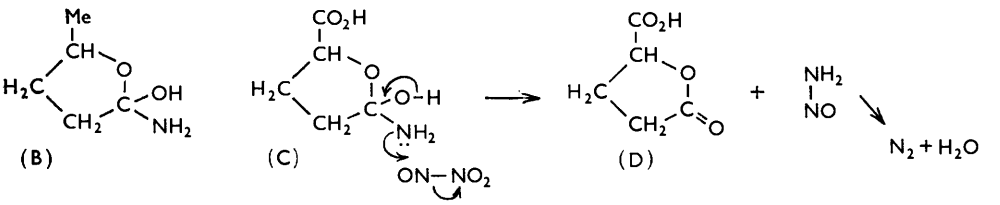


[16] Whitmore, *J. Amer. Chem. Soc.*, 1932, **54**, 3274.

With intermediate concentrations of nitrous acid varying amounts of nitrogen were obtained (Table 1), and these findings are considered to be due to the occurrence, under these conditions, of differing proportions of the above two routes.

In our view amide groups are unreactive towards weakly acid solutions of nitrous acid * (and electrophilic reagents in general) because quantum-mechanical delocalisation reduces the " availability " for bond formation of the lone-pair electrons on the nitrogen atom. In postulating that the carbonium ion derived from glutamine cyclises to form a hydroxy-amino-compound it is assumed that the electronic environment of the nitrogen atom of the original amide group has been considerably altered, that the electron-delocalisation has been removed, and that the lone-pair electrons on the nitrogen atom would be " more available " for bond formation. The hydroxy-amino-compound would be expected to be more susceptible to interaction with protons and other electrophilic reagents. It is now necessary to explain why the hydroxy-amino-compound reacts so much faster than ammonia with nitrous acid.

These hydroxy-amino-compounds, by analogy with aldehyde-ammonias, would be expected to be unstable. Moreover, Fittig [17] obtained the compound (B) by reaction of



ammonia with γ-valerolactone and found that it was readily decomposed by acids to regenerate the γ-valerolactone. The rapidity of this reaction and the ease of decomposition of aldehyde-ammonias in general indicate that very little energy is required to break down these hydroxy-amino-structures and the catalytic effect of acids almost certainly involves protonation at the nitrogen atom. Electron-accepting reagents other than protons could take part in this electrophilic attack at the nitrogen atom and if the electrophilic reagent in solution were mostly N_2O_3 one could envisage decomposition of the hydroxy-amino-compound (from glutamine) as (C) ⟶ (D), etc. A synchronous electron shift is envisaged rather than a process involving preliminary *N*-nitrosation followed by decomposition, and in this way the reaction would be analogous to the *E*2 mechanism for olefin formation.[18] The driving force for the reaction would be the stability of the carbonyl compound and the fact that the fragmentation enables interaction (nitrosation) to occur at the nitrogen atom without involving the sp^3 lone-pair electrons. The electron configuration of the nitrogen atom is accordingly not disturbed and the nitrogen is at all times in its most stable tervalent form. The reason why the hydroxy-amino-compound reacts with nitrous acid more readily than ammonia reacts is thus seen to depend on the fact that ammonia cannot augment its electron-availability at the nitrogen atom in the manner permitted by the synchronised decomposition, and its nitrosation requires all four sp^3-orbitals of the nitrogen to be utilised for bond formation.

The entities formed in the fragmentation are the lactone and $NH_2{\cdot}NO$. The latter compound would readily change to HN=N·OH and this would rapidly decompose with evolution of nitrogen. In the absence of an appreciable concentration of nitrous acid the

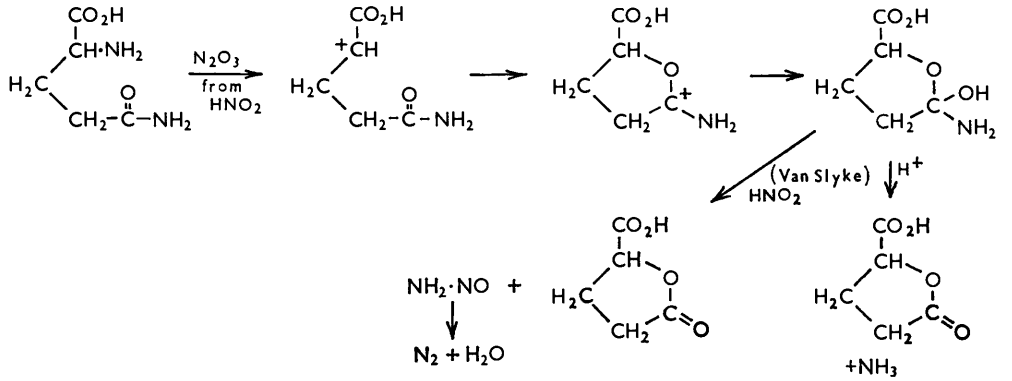
* The catalytic effect of mineral acid is considered to arise from the formation, under these conditions, of the more powerful nitrosating entities ^+H_2ONO and NO^+. The possibility that the catalytic effect is due to conversion of the amide into the imido-form, with nitrosation occurring by attack on the molecular 2π-electrons cannot, however, be excluded.

[17] Fittig, *Annalen*, 1890, **256**, 150.
[18] Ingold, " Structure and Mechanism in Organic Chemistry," Bell, London, 1953, p. 420.

decomposition of (C) would be catalysed only by protons, and the products under these conditions would then be the lactone and free ammonia.

In none of the reactions of glutamine (or glutamic acid) was the yield of lactone higher than 93%. As lactonisation of the hydroxy-acid had been shown to be slow it was thought that this figure indicated that only 93% of the carbonium ions formed in the deamination underwent cyclisation (the other 7% were probably shared between reactions leading to the hydroxy-acid or αβ-unsaturated acids, and these were not followed). It is a consequence of the statements just made that, at high concentrations of nitrous acid, the volume of nitrogen evolved in the glutamine–nitrous acid reaction would be derived as follows: Volume of nitrogen = 50% from the α-amino-group + 93% of 50% from the original amide group = 96·5%. This figure agrees satisfactorily with the experimental value of 98% (Table 2).

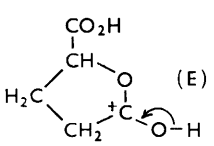
It is also consistent with the postulate of rapid and extensive cyclisation that the gas evolved in the glutamine–nitrous acid reaction is pure nitrogen, for the 93% cyclisation would preclude any but a very small amount of intervention by nitrite ions (see below).



The scheme suggested for the nitrous acid deamination of glutamine is as annexed. Deamination of γ-*N*-methylglutamine would involve a similar sequence of reactions, and in the nitrous acid reaction of the hydroxy-amino-compound the detached nitrosated fragment would be Me·NH·NO which would rapidly decompose to molecular nitrogen *via* the diazonium intermediate.

Deamination of glutamic acid would also follow the same sequence as far as the cyclic carbonium ion at which stage the product is probably formed by simple deprotonation (E).

The anomalous behaviour of glycine in the Van Slyke determination has been shown to be caused by interaction of nitrite ions with the carbonium ions which are formed in the deamination,[19] and the results for the deamination of alanine and isoglutamine indicate that similar intervention of nitrite occurs in these cases. Nitrolic acids were formed in solution, the evolved gases contained carbon dioxide, and further gas was obtained when the solutions were heated after the initial gas evolution had subsided.



(E)

None of these phenomena occurred with gutamine or glutamic acid. Intervention by nitrite ion can be ruled out. The anomaly with glutamine is related to the cyclisation, and in this way differs from the anomalous behaviour of glycine in the Van Slyke determination. Although twice as much as the expected amount of nitrogen is obtained in the deamination of glutamine the reaction is in a sense very much less disturbed than is that with glycine and the other amino-compounds noted above, for it is possible to obtain good yields of the hydroxy-acid by hydrolysis of the lactone that is formed in 93% yield.

[19] Austin, *J.*, 1950, 149.

Experimental

Glutamine.—Tosylglutamic acid was prepared by Harington and Moggridge's method [20] but in our hands the yields were not good. Excellent yields (85%) were, however, obtained by leaving the clear solution obtained by shaking the acyl chloride with glutamic acid at 70° for 2 hr. before acidification. The tosylglutamic acid was converted into glutamine according to the directions of Swan and du Vigneaud,[21] and this was purified by passage of its 0·02M-aqueous solution through a column (32 × 2·5 cm.) of cation-exchange resin, Zeo-Karb 216 (20—60 mesh). The overall yield from glutamic acid was 24% of glutamine, m. p. 182° (Found: C, 40·85; H, 6·75; N, 19·05. Calc. for $C_5H_{10}N_2O_3$: C, 41·1; H, 6·8; N, 19·2%).

γ-Carboxy-γ-butyrolactone.—Glutamic acid (14·7 g., 0·1 mole) was suspended in water (100 c.c.) at room temperature. Sodium nitrite (8·4 g., 0·12 mole), in water (60 c.c.), and 2N-sulphuric acid (60 c.c., 0·06 mole) were simultaneously added during 2 hr. The clear solution was left overnight and then evaporated under reduced pressure to a syrup. The syrup was eluted with hot acetone and, after removal of the acetone, the eluate was distilled and the fraction of b. p. 156—160°/0·2 mm. collected. On storage this slowly changed to a waxy solid, m. p. 50° (lit.,[22] 50°) (Found: C, 45·75; H, 4·55. Calc. for $C_5H_6O_4$: C, 46·2; H, 4·6%). Rapid titration with alkali (phenolphthalein) showed the presence of one carboxyl group per molecule.

γ-N-*Methylglutamine.*—This was prepared from 5-oxopyrrolidine-2-carboxylic acid and methylamine (cf. Lichtenstein [4]) and had m. p. 188° (lit.,[4] 190°) (Found: C, 44·95; H, 7·7; N, 17·2. Calc. for $C_6H_{12}N_2O_3$: C, 45·0; H, 7·5; N, 17·5%).

Isoglutamine.—The product obtained by Swan and du Vigneaud's method [21] contained much inorganic impurity; this was removed by the use of the cation-exchange resin Zeo-Karb 216. The final product had m. p. 186—187° (lit.,[21] 186°) (Found: C, 40·7; H, 7·2; N, 18·8. Calc. for $C_5H_{10}N_2O_3$: C, 41·1; H, 6·85; N, 19·2%).

Alanine Amide.—Alanine (10 g., 0·1 mole) was suspended in absolute ethanol (100 c.c.), and dry hydrogen chloride passed in until the solid dissolved. Next morning the alcohol was removed under reduced pressure and the colourless syrup obtained was treated at 0° with methanol (10 c.c.) saturated with ammonia. Ether (100 c.c.) was then added, ammonium chloride filtered off, and the solution dried (K_2CO_3) and evaporated under reduced pressure at $<20°$. The ester was dissolved in dry ether, further dried ($MgSO_4$), and distilled. The fraction of b. p. 62°/25 mm. was collected and portions (8 g.) were heated in ammonia (100 c.c.; *d* 0·88) at 70° for 24 hr. (sealed tube). The excess of ammonia was removed under a vacuum, the white residue triturated with hot chloroform, and the unchanged alanine filtered off. Evaporation of the chloroform extract gave a white amide which, recrystallised from chloroform, had m. p. 68° (lit.,[23] 72°).

Identification of the Lactone formed in the Glutamine–Nitrous Acid Reaction.—Glutamine (1 g.) in water (100 c.c.) was treated with a slight excess of sodium nitrite and 2N-sulphuric acid. After 1 hr. the solution was evaporated *in vacuo* and the solid obtained was extracted with ether for 24 hr. Removal of the ether gave a yellowish oil mixed with crystals. The infrared spectrum (in chloroform) was identical with that of authentic γ-carboxy-γ-butyrolactone prepared from α-hydroxyglutaric acid. The infrared spectra were obtained with a Grubb–Parsons double-beam spectrophotometer.

Estimation of Glutamine.—The ninhydrin colorimetric method for amino-acids was unsuitable for accurate determinations required in the kinetic runs owing to interference caused by ammonia. The copper complex method of Pope and Stevens,[24] as modified by Kay and Mills,[25] was used to estimate glutamine. The action between nitrous acid and glutamine was stopped at pH 9 (required for optimum ammine formation), and the addition of sulphamic acid to the acidified copper ammine solution destroyed any nitrous acid in solution. The estimation had an accuracy of ±2% (which sufficed) and was unaffected by the lactone in solution. The same procedure was followed in the estimation of glutamic acid.

[20] Harington and Moggridge, *J.*, 1940, 706.
[21] Swan and du Vigneaud, *J. Amer. Chem. Soc.*, 1954, **76**, 3110.
[22] Ingold, *J.*, 1921, **119**, 318.
[23] Königs and Mylo, *Ber.*, 1908, **41**, 4432.
[24] Pope and Stevens, *Biochem. J.*, 1939, **33**, 1070.
[25] Kay and Mills, *Analyt. Chem.*, 1950, **22**, 706.

Estimation of γ-Carboxy-γ-butyrolactone.—The colorimetric method using the ferric–hydroxamic acid complex [26] was adapted as follows. Samples (2 c.c.) of reaction solution were run into 0·4M-sulphamic acid (2 c.c.), to destroy nitrous acid, and after 2—3 min. alkaline hydroxylamine (2 c.c.), prepared by mixing equal volues of 2N-hydroxylamine hydrochloride and 3·5N-sodium hydroxide, were added. The hydroxamic acid was formed completely in 3 min. The solution was then acidified with 20% hydrochloric acid (1 c.c.), and 0·74M-ferric chloride (1 c.c.) was added. The red solution was diluted with 20 c.c. of 0·74M-ferric chloride in 0·1N-hydrochloric acid, and the optical density compared with that of the control determination, a Hilger photo-electric absorptiometer being used with filter no. 605. Calibration was obtained with a colour standard which was prepared from known amounts of γ-carboxy-γ-butyrolactone; the calibration chart obtained by dilution of the standard showed a linear relationship (up to 0·03M).

The lactone formed in the kinetic runs underwent less than 5% of hydrolysis in 4 hr. and equilibrium between the lactone and the hydroxy-acid required 3 days when 32% of hydroxy-acid was present. Glutamine in solution had no effect on the optical densities.

Kinetic Determinations.—The reaction vessel consisted of a 250 c.c. bolt-head flask fitted with a mercury-seal stirrer and a sampling tube that dipped below the surface of the liquid. A weighed amount of the amino-compound was dissolved in the appropriate volume of water in the reaction vessel which was then filled with pure nitrogen. The nitrogen was displaced by nitric oxide (prepared and purified by Farkas and Melville's method [27]), it having been shown that an atmosphere of nitric oxide prevents decomposition of the nitrous acid during a run. Calculated volumes of sodium nitrite solution and sulphuric acid (each having been deoxygenated under nitrogen at the reaction temperature) were rapidly introduced into the reaction flask through the sampling tube, which was then closed. Samples (2 c.c.) were withdrawn at timed intervals and the unchanged amine and the lactone estimated as described. The temperature was 24·60° ± 0·02°.

Rate Constant for Lactonisation of α-Hydroxyglutaric Acid.—Known amounts of γ-carboxy-γ-butyrolactone were dissolved in an excess of standardised alkali and left until the hydroxamic acid test for lactones was negative. The alkaline solutions were then acidified with calculated amounts of acid and the rate of lactonisation measured colorimetrically by the ferric–hydroxamic acid reaction. A Cambridge pH-meter was used to determine the pH at the onset of each run. The method of initial rates and the velocity expression gives, for $v = k[\text{Hydroxy-acid}][H^+]$, $k = 2{\cdot}95 \pm 0{\cdot}03 \times 10^{-5}$ mole^{-1} l. sec.$^{-1}$ at 24·6°.

The Van Slyke apparatus was the manometric type supplied by Messrs. Gallenkamp & Co. Ltd., London, and the detailed gas analyses were carried out on gas collected directly from the reaction vessel and submitted to selective absorbents in a series of connected gas-burettes.

One of us (J. H.) thanks the Department of Scientific and Industrial Research for financial support.

School of Chemistry, The University, Leeds, 2. [*Received, January 16th*, 1961.]

[26] Hestrin, *J. Biol. Chem.*, 1949, **180**, 249.
[27] Farkas and Melville, " Experimental Methods of Gas Reactions," MacMillan, London, 1939, p. 163.