CCXI. THE LIBERATION OF ELEMENTARY NITROGEN BY BACTERIA.

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(Received October 19th, 1931.)

THE loss of nitrogen during the putrefaction of organic matter has been the subject of numerous investigations during the past 100 years. In a review of the literature of the subject by Jensen [1906] there appears to be a difference of opinion as to the source of the nitrogen loss and of the conditions which give rise to it. Thus Reiset [1856], Dehérain and Dupont [1898] and Gibson [1895] found that nitrogen was a definite end-product in the putrefaction of meat. Lawes, Gilbert and Pugh [1861] also found that nitrogen was evolved in the aerobic decomposition of vegetable matter, and Schloesing [1889, 1] tried to confirm this result but not very successfully.

This conclusion was not supported by the work of Hüfner [1876], Kellner and Yoshi [1888], Tacke [1887] and Immendorff [1892]. These authors found that the liberation of free nitrogen only occurred in the presence of nitrites or nitrates. Jensen is of opinion that the results of the earlier workers are not reliable owing to faulty analytical methods.

Löhnis [1926] states that a loss of nitrogen from organic matter occurs only in the presence of air, and that it has not yet been proved that free nitrogen is derived from the amino-group or ammonia. He suggests the possibility of a liberation of nitrogen by the purely chemical interaction between amines or NH_3 and nitrous acid thus:

This possibility was first put forward by Dietzell [1882], though the reaction was first discovered by Piria [1849]. Fowler and Kotwal [1924] found it to be ineffective except in the presence of mineral acid. Grimberty [1899], however, suggested that a liberation of amino-nitrogen may occur in a nitrate-broth medium without the development of acidity or perhaps of only local and temporary acidity.

Russell [1927] agrees with Jensen as to the unreliability of the analytical methods used by the earlier workers but considers the results of Lawes, Gilbert and Pugh [1861] and of Pfeiffer *et al.* [1897] as sufficient evidence of the possibility of nitrogen being a final product of protein breakdown. In collaboration with Richards [1917], however, he showed that no nitrogen

was evolved in the decomposition of dung under completely aerobic or completely anaerobic conditions but only under a combination of the two conditions. He accounted for the result of Lawes, Gilbert and Pugh and others by the formation under anaerobic conditions of unstable intermediate products which evolve nitrogen on contact with air. Waksman [1927] in his review of the literature does not refer to the possibility of the liberation of free nitrogen without denitrification.

Since the work of Lawes, Gilbert and Pugh, and of Pfeiffer on the liberation of nitrogen from organic matter by oxidation, three authors have recorded the same phenomenon under anaerobic conditions. Wood and Willcox [1897] obtained considerable quantities (150 cc.) of nitrogen from a peptone-glucose medium inoculated with a pure culture of a bacillus isolated from fermenting bran used in the bating of hides. Unfortunately this experiment is not above criticism since oxygen gas was also collected, which, as a product of anaerobic fermentation, suggests the leakage of air into the apparatus. Harden [1901], using a peptone-glucose medium inoculated with *B. coli*, obtained a small amount of nitrogen (7.5 cc.) but none in absence of glucose. The rôle of the sugar in promoting the evolution of nitrogen is somewhat obscure, since carbohydrates are known to have a depressing effect on ammonification [Doryland, 1916], which would presumably be an intermediate stage in the formation of free nitrogen. Bach and Sierp [1923], using sewage sludge, obtained considerable quantities of nitrogen during anaerobic (?) fermentation in the presence of large volumes of tap-water. As pointed out by Buswell and Neave [1927] the results are subject to a very large error from the daily exchange of 500 cc. of tap-water containing sufficient dissolved air to account for all the nitrogen collected.

Thus, the evidence amply bears out Pfeiffer's remarks that "there is scarcely another field of research in agricultural chemistry in which we encounter contradictions so numerous and so fully unexplained."

As Jensen points out, the position regarding the formation of free nitrogen by bacteria is complicated, not only as regards the source of the nitrogen but also as to the conditions which give rise to it.

Amidst the conflicting evidence of previous workers there is one process of the liberation of free nitrogen by bacteria that is beyond question, *viz.* denitrification. According to Mair [1908], Beijerinck and Minkman [1910] and Lloyd and Cranston [1930], nitrates are reduced in three stages: (1) nitrite, (2) hyponitrite, (3) free nitrogen. It follows therefore that in all attempts to prove the liberation of nitrogen by the oxidation of amines or ammonia it is essential to ensure the absence of nitrates, nitrites and hyponitrites. The presence of nitrifying bacteria during putrefaction has been well established by numerous workers. These organisms are now recognised as taking part in biological oxidation in symbiosis with heterotrophic organisms [Mair, 1908; Barritt, 1931].

Ammonification studies have been carried out by numerous workers in

recent years, and the production of ammonia is now regarded as an essential part of the bacterial oxidation of organic matter when the C/N ratio of the substrate is not too high [Marschal, 1893; Doryland, 1916]. The yield of ammonia is quantitative and no recent worker has recorded a loss of free nitrogen. The only certain products of the biological oxidation of ammonia are nitrite and nitrate, and, since a crude inoculation of soil organisms will oxidise ammonia quantitatively to nitrous or nitric acid [Schloesing, 1889, 2], there is no reason to suppose the existence in soil of organisms capable of oxidising ammonia to free nitrogen.

These observations are amply borne out by the work of Russell and Richards [1917] on the loss of nitrogen during the decomposition of manure, and by the work of the Royal Commission on Sewage Disposal [1908]. In both cases loss of nitrogen was only recorded in the presence of nitrates, which represent the balance of the nitrification process in excess of denitrification as stated above.

Since denitrification appears to be an essential part of the process, the following investigations were undertaken with a view to obtaining more precise data on this process and also on the possible interaction of amines and ammonia with nitrous acid, a subject which has received little attention from previous workers. The experiments of Fowler and Kotwal [1924] on this point involved small gasometric measurements (2 cc.) and are not at all convincing.

EXPERIMENTAL.

Culture solutions of 0.5 % concentration of the following substances were prepared with the addition of 0.05 % potassium phosphate, 0.03 % sodium chloride and 0.1 % potassium nitrate: urea, uric acid, glycine, alanine, tyrosine, ammonium acetate, ammonium sulphate, dextrose + ammonium sulphate, dextrose, acetic acid and alcohol. The distilled water used was previously inoculated by shaking each litre with 1 g. of Broadbalk soil. The initial p_H was adjusted to 6.5. 200 cc. of each culture were incubated at 25° in a 300 cc. stoppered bottle for 6 weeks. Determinations of organic *plus* ammonia nitrogen were made by the Kjeldahl method, nitrites by the Griess-Ilosvay method and nitrate by the pyrogallol colorimetric method.

During the incubation period the reaction of the culture solutions was tested daily. The rise in p_H due to ammonification of organic nitrogen and liberation of alkali by the denitrification of the potassium nitrite was corrected by the addition of solid KH_2PO_4 , and the lowering of p_H due to glycolysis was corrected to some extent by the addition of calcium carbonate. In this way an average p_H of 6.5 was maintained.

The formation of nitrite occurred in all cultures within 24 hours and persisted until all the nitrate had disappeared. The amount of nitrite produced varied with the different solutions, the greatest amounts (0.013 %) being recorded in the solutions containing dextrose and alcohol. The nitrite also per-

sisted in these solutions throughout the whole period of 6 weeks. The most vigorous reduction occurred in the solutions of uric acid and amino-acids, in which cases the amount of nitrite never exceeded 0.003 % and completely disappeared within 24 days. The slower rate of reduction in the carbohydrate solution is probably due to the lower p_H owing to the buffering action of the calcium carbonate being arrested by a covering of bacterial growth.

The formation of organic acids from dextrose does not require oxygen and the oxidation of these acids is very much reduced at $p_H < 6$ [Cook and Alcock, 1931]. For this reason the nitrite is more slowly reduced and therefore persists in the carbohydrate solutions for a much longer period than in the solutions containing amino-compounds. The results of the nitrogen estimations are given in Table I.

Table I.

Substance	C/N ratio	Initial N g. per 100 cc.		Final N after incubation			Loss of N	
		organic N + NH ₃	as N ₂ O ₅	organic N + NH ₃	N ₂ O ₃	as N ₂ O ₅	organic N + NH ₃	as N ₂ O ₅
1 Ammonium sulphate	—	0.053	0.014	0.0531	Trace	0.014	Nil	Nil
2 Urea	0.43	0.233	0.014	0.235	Trace	0.014	Nil	Nil
3 Uric acid	1.07	0.167	0.014	0.166	—	—	0.001	0.014
4 Glycine	1.71	0.0933	0.014	0.0928	—	—	0.0005	0.014
5 Ammonium acetate	1.71	0.0530	0.014	0.0523	—	—	0.0007	0.014
6 Alanine	2.57	0.0777	0.014	0.0776	—	—	0.0001	0.014
7 Tyrosine	7.7	0.0379	0.014	0.0372	—	—	0.0007	0.014
8 Dextrose + (NH ₄) ₂ SO ₄	10.0	0.0127	0.014	0.0124	—	0.0136	0.0003	0.0004
9 Dextrose only	14.5	—	0.014	0.00501	—	—	(increase 0.00501)	0.009
10 Acetic acid	14.5	—	0.014	0.00093	0.004	0.009	(increase 0.00093)	0.0007
11 Alcohol	18.0	—	0.014	0.00125	—	—	(increase 0.00125)	0.0127
12 Glycine + dextrose	3.5	0.0933	0.014	0.0916	0.0025	—	(loss 0.0017)	0.0115

It will be seen that there is no significant loss of organic nitrogen or ammonia in any of the solutions except No. 12 (glycine + dextrose), in which case a loss of 0.0017 g. nitrogen occurs. Comparing this result with that of No. 4 (glycine alone) it appears that the addition of dextrose to culture No. 4 (glycine alone) has resulted in a loss of organic nitrogen.

In the case of cultures Nos. 1 and 2, ammonium sulphate and urea, there was also no loss of nitrate nitrogen. Ammonification of the urea occurred, but no further change.

In cultures Nos. 3, 4, 5, 6 and 7 (of low C/N ratio) denitrification was complete in 21 days, and although these were incubated for a further period of 21 days no further loss of nitrogen occurred. Therefore the organisms concerned in the liberation of nitrogen during the first 3 weeks were unable to liberate nitrogen from either ammonia or organic nitrogen.

The higher C/N ratios of cultures Nos. 9, 10 and 11 appear to be associated with a smaller loss of nitrate nitrogen and a slight increase in organic nitrogen due to synthesis.

The loss of nitrogen in culture No. 12 appears at first sight to confirm Harden's observation of the liberation of nitrogen from protein in the presence of sugar. In the above case however nitrites were present, in which respect it differs from Harden's unless nitrates or nitrites were present as an impurity. Considerable amounts of lactic acid were formed in Harden's culture with glucose and it is well known that nitrites may react with amino-acids in presence of organic acid (*e.g.* acetic acid in Van Slyke's method of estimating amino-N). We may therefore expect a loss of amino-nitrogen in the presence of organic acids and nitrite. If nitrite was present in Harden's culture, free nitrogen should also have been formed in the peptone solution without glucose by the denitrification process. In this case however the amount of nitrogen liberated would be exactly half that formed by the interaction of amines with nitrous acid. Possibly 3.5 cc. of nitrogen is not sufficient for measurement with any degree of accuracy [see Russell and Richards, 1917]. In the cases where nitrite is present at the end of the fermentation the recorded loss of organic nitrogen might have occurred by means of the interaction between amines and nitrous acid on adding sulphuric acid for the Kjeldahl digestion. The possible loss of nitrogen at this stage was tested by adding excess nitrite to a 0.5 % solution of glycine before the acid digestion. The final organic nitrogen was identical with that of the materials without the glycine, indicating complete decomposition of the amino-group. In culture No. 12 therefore the recorded loss of organic nitrogen may have occurred during analysis and not during the fermentation.

In order to test the possibility of this reaction occurring during acid fermentation sterile solutions of glycine and sodium nitrite were prepared and varying quantities of lactic acid added. The amino-nitrogen was determined by Sørensen's method of formaldehyde titration. The results obtained are given in Table II.

Table II.

	Initial p_H	cc. N/10 NaOH required
0.5 % glycine + 0.5 % NaNO_2	6.5	31.0
" " + lactic acid	6.2	30.6 (12 hrs.)
" " "	5.7	30.5 "
" " "	4.8	30.5 "
" " "	4.2	28.4 "
" " "	3.8	25.8 (5 mins.)
		7.8 (12 hrs.)

These results show that the rate of decomposition of the amino-acid is considerably diminished above p_H 4. It was afterwards found that the p_H of the less acid solutions increased rapidly, so that an initial p_H of 4.2 became 5.2 in 10 minutes and 6.5 in 1 hour. This rise in p_H is probably due to the volatility of the nitrous acid. At $p_H < 5.0$ a rapid evolution of gas occurred only on shaking the flask, and no gas appeared to be evolved when the solution remained quiescent. This liability to supersaturation with nitrogen gas must invalidate the results obtained by gasometric methods unless accompanied

by vigorous fermentation. With a continuous production of organic acid such as occurs in the fermentation of carbohydrates a more effective interaction between the amino-acid and nitrites could occur and result in a greater loss of free nitrogen.

Under strongly aerobic conditions organic acids are oxidised to carbonic acid with a consequent rise in p_H of the medium but, since nitrous acid is also liberated from nitrites by carbon dioxide [Moore, 1904], it seemed desirable to repeat these experiments with carbonic acid in place of lactic acid. Solutions of carbonic acid were obtained from a sparklet-siphon, using distilled water and liquid CO_2 . A solution of calcium bicarbonate was also used. The results obtained are as follows:

Formaldehyde titrations of 0.5 % glycine + 0.5 % nitrite

	p_H	cc. N/10 NaOH required
In water only	6.8	31.0
In supersaturated solutions of CO_2 at atmospheric pressure 12 hours	5.8	23.2
In saturated solution of CO_2 under pressure	5.8	20.5
In calcium bicarbonate solution	6.0-6.8	30.0

These results show that it is possible to obtain a loss of nitrogen by the interaction of glycine and nitrite in a solution supersaturated with carbon dioxide. It is unlikely that such conditions would occur in the decomposition of organic matter. In the culture solutions described above (Table I) the reaction was maintained at p_H 6.5 and no loss of amino-nitrogen occurred [cf. Grimbert, 1899].

From these results it must be concluded that a loss of organic nitrogen is possible during denitrification provided that organic acids and amino-compounds are simultaneously produced. This event is more likely to occur when readily available carbohydrates like dextrose are present. It would also be expected to occur in the absence of nitrates when anaerobic conditions are followed by aeration. In the presence of nitrifying organisms amino-acids may be regarded as the unstable compounds referred to by Russell and Richards [1917] as oxidising to elementary nitrogen on exposure to air.

The simultaneous production of ammonia by the oxidation of protein and of nitrite by the reduction of nitrate suggests the possibility of free nitrogen being formed by the spontaneous decomposition of ammonium nitrite. This has been suggested by both Löhnis and Waksman. It is well known that ammonium nitrite is readily decomposed on heating, but in dilute solution it appears to be quite stable. A 0.5 % solution accurately prepared by mixing molecular equivalents of ammonium sulphate and sodium nitrite was incubated at 25° for 30 days without loss of either ammonia or nitrite and without a trace of gas being produced. The same result was obtained when the solution was incubated with soil. It must therefore be concluded that the decomposition of ammonium nitrite is not a source of the liberation of free nitrogen by bacteria.

SUMMARY.

1. A review of the literature shows that much confusion exists regarding the liberation of nitrogen by bacteria. The evidence of some workers that it occurs under anaerobic conditions is cancelled by that of others who show that it occurs only under aerobic conditions.

2. It is pointed out that inaccuracies in analytical methods and insufficient attention to the occurrence of nitrates or nitrification is responsible for the confusion as to the nature of the process.

3. Experiments are described which confirm the results of several previous workers that

(a) free nitrogen is liberated by soil bacteria during denitrification;

(b) there is no evolution of nitrogen from organic compounds under anaerobic conditions in the absence of oxidised nitrogen, nor from oxidised nitrogen in the absence of oxidisable organic compounds.

4. The reduction of nitrates and nitrites is effected most rapidly in the presence of compounds of low C/N ratio, and the formation of free nitrogen is strictly confined to the reduction of oxidised nitrogen provided the reaction of the medium does not fall below p_H 6.5.

5. The production of free nitrogen from organic compounds does not occur by bacterial action but only indirectly by the interaction between free nitrous acid and amino-compounds, both of which may be produced simultaneously by bacterial agency.

6. Free nitrous acid is liberated from nitrites by organic acids and carbonic acid, but does not effect the decomposition of amino-acids unless the p_H of the medium is < 6.0 .

7. The presence in a culture of readily available carbohydrates may result in a loss of free nitrogen from organic matter by the indirect process referred to in 5.

8. In Kjeldahl determinations of solutions of organic nitrogen the presence of nitrite results in a loss of amino-nitrogen during the acid digestion.

9. There is no liberation of free nitrogen by the decomposition of ammonium nitrite in culture solutions at ordinary temperatures.

The writer is much indebted to Mr R. G. Warren of the Chemical Department, for much useful advice and for the control of the Kjeldahl determinations, and also to Dr L. de Telegdy Kovato of Budapest (late of this department) for help in the discussion of the literature.

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