124. THE NITROGEN CYCLE IN BIOLOGICAL SYSTEMS

2. CHANGES IN MIXTURES OF SEWAGE AND ACTIVATED SLUDGE ENTAILING THE LIBERATION OF NITROGEN

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(Received 29 April 1940)

SOME of the early work on the biological aspects of sewage purification was concerned with the nitrification processes. Mair [1908] considered the problem with special reference to denitrification, using a percolating filter in combination with contact beds. He detected hyponitrous acid during the reduction of nitrate in sewage and isolated an organism, *Bacillus hyponitrosus*, able to reduce nitrate to hyponitrite in pure culture. Mair observed also that the disappearance of nitrate, which occurred at one stage of the treatment, was accompanied by the release of N_2 and he regarded this as an instance of denitrification. Some investigators, e.g. Heukelekian & Chamberlin [1922-3] and Bach [1932], have reported losses of N during the treatment of sewage by the activated sludge process. Barritt [1931; 1933] discussed nitrification in biological filters and reviewed the literature on the subject of the liberation of N by bacteria. Bonazzi [1921] found that *Azotobacter chroococcum* effected fixation of N in a medium with or without the addition of ammonium sulphate, but a loss of N resulted from the utilization of nitrate by this organism. Korsakova [1927] showed that, during the oxidation of such carbon compounds as glucose and citric acid by *B. fluorescens liquefaciens* or by *B. pyocyaneus*, the formation of protein from the N of nitrate was accompanied by a liberation of N_2 and the intermediate production of nitrite. Richards & Shrikhande [1935], who investigated the utilization of different forms of inorganic N during the decomposition of plant materials, showed the possibility of denitrification during the conversion of nitrate- into protein-N.

In the previous paper [Corbet & Wooldridge, 1940], it was shown that, under certain conditions, and particularly with non-nitrifying mixtures of sewage and activated sludge, N was lost as gas during aeration. In this paper the problem of liberation of N is examined further, using fresh activated sludge, sterilized sludges subsequently inoculated with organisms in pure culture and chemically defined media inoculated with organisms.

Experimental technique

The technique of analysis was similar to that described in the previous paper. When necessary, the sewages and sludges and the chemically defined media were sterilized by autoclaving. A medium frequently used was Reader's

medium,¹ sometimes modified in the ways described in the text. Various micro-organisms were used but *Chromobacterium prodigiosum* and a yeast isolated from sewage were often employed. The characteristics of this yeast were studied and, as no ascospore formation was observed, it has been assigned to the genus *Torula*. This generic classification is tentative and, for purposes of reference, it is proposed to name the organism *Torula nitrivertens* *sp. nov.*

This organism,² which is a bottom yeast, grew well on the usual media, good growth being obtained on beer-wort agar overnight. The organism was obtained in pure culture by continued subculturing in beer-wort broth adjusted to pH 2.8. A thorough and repeated examination by microscopic methods (stained and unstained preparations), and by cultivation on ordinary bacteriological media, failed to reveal the presence of any bacterial contaminants. Unstained, this organism had the appearance of a typical yeast, comprising large, ellipsoid cells, which varied somewhat in size and shape according to the conditions of cultivation; chains with up to four or five cells were not rare. A stroke culture on beer-wort agar gave abundant growth. With stroke cultures on ordinary nutrient agar, and on nitrate-glucose agar, growth was moderate to abundant, beaded, convex, glistening, smooth, opaque, buff, slimy; no aerial growth. The agar colonies were round, with surface smooth and convex, and edges entire; no liquefaction of gelatin. Growth in heart broth and in nitrate-glucose solution good; in Thornton's agar (an inorganic salt mixture containing asparagine) very poor, only becoming evident after 4 or 5 days. Facultative anaerobe. No chromogenesis. After incubation for 3 days at 34°, the organism produced acid and gas with sucrose; acid and a little gas with glucose; no change with maltose, lactose and mannitol; and only slight curd formation with litmus milk. Nitrate was reduced to nitrite. It will be seen that this organism was able to liberate or fix N under the appropriate conditions. It is not, however, the first N-fixing yeast to be reported, for de Kruijff [1910] described the isolation from garden soil in Java of such an organism, which he named *Torula bogoriensis rubra*, since it formed blood-red colonies.

Experiments with sterilized mixtures of sewage and activated sludge inoculated with pure cultures of micro-organisms

When sterilized mixtures of sewage and activated sludge were inoculated with pure cultures of different organisms and were incubated for about 2 weeks at 34° there was often a significant loss of N, although no loss occurred from the uninoculated controls (Table 1). Thus, it is evident that a mixed flora is not essential to a loss of N from sewage systems. The addition of other carbon- or N-containing substances may affect the ultimate distribution of the N. The experiments given in Table 2 indicate that the loss of N from non-sterile mixtures of sewage and activated sludge can be largely or completely annulled by the addition of such substances as glucose and pyruvic acid, whereas the loss may be increased by the addition of nitrate or glycine. Sterilized mixtures of sewage and sludge inoculated with *Chromobacterium prodigiosum*, *Torula nitrivertens* or *Staphylococcus aureus* showed no significant loss of N in presence of sucrose, in contrast to the significant losses which occurred with potassium nitrate (Table 3).

Reader's medium contains 3 g. $(\text{NH}_4)_2\text{SO}_4$; 1 g. KH_2PO_4 ; 0.16 g. K_2HPO_4 ; 0.5 g. NaCl ; 0.7 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.4 g. $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$; 50 g. glucose and 1000 ml. distilled water.

² We are indebted to Miss R. Fellowes for help with the isolation of this organism.

Table 1. *Losses of N during incubation of sterilized mixtures of sewage and activated sludge inoculated with cultures of different species of micro-organisms*

The mixtures were incubated at 34° for 14 days (top table) and for 17 days (lower table).

The figures refer to mg. N in 25.0 ml. of sterilized sewage and sludge; each figure represents the mean for four flasks.

Micro-organism	Initial N content	Final N content	% loss of N	Significance of difference between initial and final N contents*		
				<i>n</i>	<i>t</i>	<i>P</i>
Uninoculated controls	4.5	4.5	—	6	—	—
<i>Staphylococcus albus</i>	8.5	8.0	5.9	6	4.62	<0.01
<i>Chromobacterium prodigiosum</i>	8.4	7.0	16.7	6	10.33	<0.01
<i>Pseudomonas pyocyanea</i>	8.3	7.2	13.3	6	9.53	<0.01
<i>Torula nitrovertens</i>	8.6	8.3	3.5	6	4.24	<0.01
Uninoculated controls	4.6	4.7	—	6	0.49	Between 0.7 and 0.6
Undetermined short rod, isolated from sewage	5.8	4.7	19.0	6	5.88	<0.01

* See Corbet & Wooldridge [1936]. $n = (N - 1) + (N' - 1)$, N and N' being the number of replicate determinations.

$$t = \frac{\bar{x} - \bar{x}'}{s} \sqrt{\frac{1}{N} + \frac{1}{N'}}$$

$\bar{x}$ and $\bar{x}'$ being the respective means and s the estimated standard deviation.

P represents the probability of the difference between the means occurring by chance. In the present investigation, differences between means are regarded as significant only when $P < 0.01$, that is, when such differences can occur by chance less than once in a hundred times.

Table 2. *Changes in the N content of mixtures of sewage and activated sludge containing added compounds, during incubation for 45 days at 29°*

The figures in the second and third columns are means of four experiments and refer to mg. N per 125 ml. of sewage and sludge mixture.

Compounds added	Initial N content	Final N content	% loss	Difference between initial and final N values		
				<i>n</i>	<i>t</i>	<i>P</i>
No addition	6.3	4.6	27.0	6	13.52	<0.01
0.25 g. glucose	6.3	5.8	7.9	6	2.87	Between 0.05 and 0.02
0.50 g. glucose	6.3	6.5	3.2 (gain)	5	1.00	Between 0.4 and 0.3
0.05 g. pyruvic acid	6.3	5.2	17.5	6	5.50	<0.01
0.10 g. pyruvic acid	6.3	6.6	4.8 (gain)	6	2.27	Between 0.1 and 0.05
0.0060 g. glycine	12.3	6.6	46.3	5	20.70	<0.01
0.0120 g. glycine	18.3	7.4	59.6	6	43.02	<0.01
0.0043 g. NO ₃ -N in KNO ₃	10.6	4.1	61.3	6	29.54	<0.01

The addition of a small inoculum of a mixture of sewage and activated sludge into Reader's medium resulted in a small loss of N, but the loss was materially increased when the ammonium sulphate (one constituent of the medium) was replaced by potassium nitrate (Table 4). Similarly, although incubation of Reader's medium inoculated with *Chromobacterium prodigiosum* or with *Torula nitrovertens* did not result in a loss of N, there was a loss of N during incubation of Reader's medium inoculated with these organisms when

Table 3. *Changes in the N content of sterilized mixtures of sewage and activated sludge containing added compounds, inoculated with pure cultures of micro-organisms*

The flasks were incubated at 34° for 17 days with *Staph. aureus*, and for 20 days with the other organisms; each test was carried out in quadruplicate. The N content is in mg. per 25.0 ml. of sterilized sewage and sludge mixture.

Addition	Initial N content	Final N content	Significance of difference between initial and final N contents		
			<i>n</i>	<i>t</i>	<i>P</i>
<i>Staphylococcus aureus</i>					
Uninoculated controls	8.5	8.2	6	1.17	Between 0.3 and 0.2
No addition	9.0	9.2	6	0.71	0.5
0.50 g. sucrose	9.0	9.1	6	0.32	Between 0.8 and 0.7
Potassium nitrate (8.3 mg. NO ₃ -N)	17.3	13.5	6	22.56	<0.01
<i>Chromobacterium prodigiosum</i>					
No addition	14.4	11.6	6	28.00	<0.01
0.48 g. sucrose	14.4	13.9	6	2.47	Between 0.05 and 0.02
Potassium nitrate (8.8 mg. NO ₃ -N)	23.2	14.2	6	39.26	<0.01
<i>Torula nitrovertens</i>					
0.48 g. sucrose	14.3	14.6	6	0.95	Between 0.4 and 0.3
Potassium nitrate (6.7 mg. NO ₃ -N)	21.0	19.9	6	6.73	<0.01

Table 4. *Changes in the N content of Reader's medium, inoculated with sewage and activated sludge*

The flasks were incubated for 10 days at 34°.

The figures in the first and second columns refer to mg. N in 25.0 ml. of Reader's medium inoculated with 1.0 ml. of sewage and activated sludge, and represent the mean value of replicated experiments.

Form of N	Initial N content	Final N content	% loss	Significance of difference between initial and final mean values of N		
				<i>n</i>	<i>t</i>	<i>P</i>
Ammonium sulphate	16.8	16.4	2.4	10	5.52	<0.01
Potassium nitrate	12.2	10.3	15.6	10	4.71	<0.01

the ammonium sulphate had been replaced by potassium nitrate (Tables 5 and 6). It will be seen in Table 5 that a small gain in N resulted in the medium containing ammonium salt. Several unsuccessful attempts were made to obtain N fixation in larger quantities, but in an experiment in which 25.0 ml. of Reader's medium containing 0.5 g. of different carbohydrates were inoculated with *Chromobacterium prodigiosum*, after 16 days at 34°, the N content increased from 11.4 to 11.9 mg. (glucose), 11.6 (fructose) and 11.9 (sucrose): in a parallel experiment with *Torula nitrovertens*, the N content increased from 11.4 to 11.7, 11.9 and 11.8 mg. respectively.

Experiments with a pure culture activated sludge

Butterfield [1937] prepared pure culture activated sludges comprising a single bacterial species; these sludges achieved a high rate of oxidation, but no account was taken of the N cycle. We carried out a few experiments with a pure culture sludge, prepared by aeration of a mixture of sterilized sewage and a little sterilized sludge, after inoculation with *Chromobacterium prodigiosum*;

Table 5. *N* changes during incubation of Reader's medium, inoculated with *Chromobacterium prodigiosum*

The figures refer to mg. N in 25.0 ml. of medium, and each result represents the mean N content of four flasks.

(a) Reader's medium with ammonium sulphate¹

(i) Initial N content	(ii) N content after 14 days at 34°	(iii) N content after 21 days at 34°
34.1	34.4	34.8
Between (i) and (ii), $n=6$, $t=2.22$, P between 0.1 and 0.05.		
Between (i) and (iii), $n=6$, $t=3.74$, $P < 0.01$.		

(b) Reader's medium with potassium nitrate

(i) Initial N content	(ii) N content after 21 days at 34°
14.3	9.8

Between (i) and (ii), $n=6$, $t=5.88$, $P < 0.01$.

The flasks contained 7.5 mg. nitrate-N at the beginning of the experiment, and 6.6 mg. nitrite- and nitrate-N at the close, nitrite being present in all four flasks.

Table 6. *Changes in the distribution of N compounds in Reader's medium inoculated with Torula nitrivertens during incubation at 34°*

The figures refer to mg. N in 25.0 ml. of medium

(a) Reader's medium with ammonium sulphate

Days	Organic N	NH ₃ -N	NO ₂ -N	NO ₃ -N	Total N	Gain (+) or loss (-) of N
—	0.0	11.3	0.0	0.0	11.3	—
1	1.0	10.3	0.0	0.0	11.3	—
4	1.7	9.6	0.0	0.0	11.3	—
8	2.0	8.8	0.0	0.0	10.8	-0.5
11	3.8	8.0	0.0	0.0	11.8	+0.5
15	3.7	8.1	0.0	0.0	11.8	+0.5
23	3.8	8.0	0.0	0.0	11.8	+0.5
28*	3.7	8.2	0.0	0.0	11.9	+0.6
45	4.2	7.5	0.0	0.0	11.7	+0.4

* On the 25th day, 10.0 ml. of sterilized 5% glucose solution were added to each of the remaining two flasks.

(b) Reader's medium with potassium nitrate

Days	Organic N	NH ₃ -N	NO ₂ -N	NO ₃ -N	Total N	Loss of N
—	0.2	0.0	0.0	7.9	8.1	—
2	2.1	0.0	0.1	5.6	7.8	0.3
3	1.4	0.0	0.0	4.9	6.3	1.8
7	2.0	0.0	0.0	4.1	6.1	2.0
9*	2.1	0.0	0.0	5.0	7.1	1.0
16	3.2	0.0	0.0	3.2	6.4	1.7
22	3.7	0.0	0.0	3.1	6.8	1.3

* On the 7th day, 10.0 ml. of a sterilized 5% glucose solution were added to each of the remaining three flasks.

In a parallel experiment, the gas evolved after 3 days' incubation in an apparatus in which one limb was open to the air was collected by displacement and analysis showed it to consist of 24% N and 76% CO₂.

aeration was continued for several weeks through a number of changes of sterilized sewage. In a subsequent experiment, the purification of sterilized sewage by the small volume of *Chr. prodigiosum* sludge thus obtained was

¹ To ensure good growth, this medium was prepared to contain 1% of peptone.

followed by determination of the changes in the N distribution and in the biochemical oxygen demand (Table 7). This experiment, which was carried out in a 1 l. flask, was conducted as in Table 2 of the previous paper, except that 50.0 ml. samples were taken for the N analysis and B.O.D. determinations at the intervals stated. As the organism used was not a nitrifier, no nitrification occurred of any ammoniacal N present in excess of synthetic requirements.

Table 7. *Treatment of sewage by aeration in admixture with a Chromobacterium prodigiosum sludge*

The figures are calculated as mg. N or (in the case of B.O.D.) oxygen in 100 ml. of the mixture (that is, the figures refer to parts per 100,000).

Period of aeration	Supernatant liquid					Moist solid Total N	Total N in both phases
	NH ₃ -N	NO ₂ -N	NO ₃ -N	Organic N	B.O.D.		
Beginning	3.1	0.0	0.0	0.5	18.4	1.0	4.6
4 hr.	2.7	0.0	0.0	0.9	18.2	0.7	4.3
1 day	2.3	0.0	0.0	1.1	13.6	1.1	4.5
2 days	1.7	0.0	0.0	0.9	9.6	0.9	3.5
5 days	1.8	0.0	0.0	1.1	12.7	1.6	4.5

During the 5 days' aeration 2.0 mg. of ammonia-N were liberated. A calculation showed that the contents of the aeration flask (430 ml.) contained 19.8 mg. of N initially, of which 10.7 mg. were removed in samples and 2.0 mg. as ammonia-N, and that no losses of N₂ had occurred.

In a further experiment, mixtures of *Chr. prodigiosum* sludge and sterilized sewage were aerated in a series of glass flasks with additions of ammonium sulphate, potassium nitrite or potassium nitrate (Table 8). In the control

Table 8. *Effect on sewage of aeration in admixture with a Chromobacterium prodigiosum sludge, in the presence of added nitrogenous salts*

The incoming air was ammonia-free (passage through sulphuric acid), and the ammonia-N liberated from the sewage material in the flasks during the 5 days' aeration was collected and estimated.

The figures refer to mg. N contained in the flasks and each figure represents the mean of two experiments.

Form of N added to 100.0 ml. sterilized sewage and 10.0 ml. of <i>Chr. prodigiosum</i> sludge	Before aeration					After aeration					Loss of N
	NH ₃ -N	NO ₂ -N	NO ₃ -N	Organic N	Total N	NH ₃ -N	NO ₂ -N	NO ₃ -N	Organic N	Total N	
No addition	2.5	0.4	0.0	3.3	6.2	0.4	0.1	0.0	4.7	5.2	1.0
Ammonium sulphate (5.2 mg. N)	7.7	0.4	0.0	3.3	11.4	2.8	0.0	0.0	6.2	9.0	2.4
Potassium nitrite (5.0 mg. N)	2.5	5.4	0.0	3.3	11.2	0.2	4.5	0.0	4.9	9.6	1.6
Potassium nitrate (4.0 mg. N)	2.5	0.4	4.0	3.3	10.2	0.7	0.2	3.6	4.3	8.8	1.4
Total loss of N											6.4
N liberated as NH ₃ -N											1.5
N presumed lost as gas											4.9

flasks without any additions, there was a conversion of ammoniacal N into organic form together with some loss of N. The addition of ammonia-N resulted in a greater production of protein and a loss of some ammonia in the air stream: the added nitrite or nitrate failed to increase the amount of organic N produced. The

results obtained in these experiments with a pure culture sludge are in accord with the work already described with natural sludges, under conditions in which no nitrification took place.

Rising of activated sludge

Normally, in an activated sludge plant the particles of sludge can readily be settled in a sedimentation tank, but, on occasion, they may rise to the surface, being buoyed up by bubbles of gas which have been found to be N [O'Shaughnessy & Hewitt, 1935]. A number of investigators [e.g. McLachlan, 1936, and Bragstad & Bradney, 1935] observed that rising of sludge was accompanied by the presence of nitrite.

Our experience, which is based upon data from laboratory experiments, has been that nitrite formation can occur with both nitrifying and non-nitrifying mixtures of sewage and sludge. With the former, oxidation from nitrite to nitrate is usually rapid, but, if it is sufficiently slow to allow an accumulation of nitrite, rising of sludge may take place on the cessation of aeration. In these circumstances rising of sludge is not the result of an anaerobic denitrification.

Occasionally, a non-nitrifying sewage and sludge mixture may give rise to nitrite, followed by an accumulation of this compound and rising of the sludge. Tests have been carried out showing that, with such a non-nitrifying mixture, rising can be initiated or aggravated by addition of nitrite or nitrate, or inhibited by addition of assimilable carbonaceous material (glucose or soluble starch). Our results suggest that the occurrence of rising is dependent upon nitrite formation being in progress while some organic matter remains in the sewage, for it appears that N liberation occurs principally during the utilization of nitrite for protein synthesis.

Chemical reactions entailing the decomposition of nitrite

Although small losses of N may occur in sewage and sludge mixtures during aeration, or on prolonged incubation, such losses are usually slight. As the considerable N losses reported in these papers were almost invariably associated with the presence of nitrite and, in some instances, were proved to be due to the release of elementary N, experiments were carried out to ascertain to what extent any loss of N might be due to chemical decomposition of nitrite.

Continuous aeration of mixtures of sewage and sludge containing added nitrite, under conditions of sterility, produced very little disruption of the nitrite, although there was a slight loss of N from the sewage and sludge (Table 8 of previous paper). Similarly, the presence of added ammonia- and nitrite-N together did not result in a loss of nitrite if the conditions were maintained sterile (Table 9).

Except under acid conditions, where there is a chemical oxidation of nitrite to nitrate, the extent of which depends upon the pH value of the medium [Corbet, 1934], ammonium nitrite is stable and can be preserved in the dark for some months without change [Corbet, 1935]. In the present investigation, it was found that no loss of N gas occurred when ammonium sulphate and potassium nitrite were boiled together with magnesia and Devarda's alloy, the nitrite being reduced quantitatively to ammonia. The loss of N as ammonia from the sewages during aeration by purely chemical agency was usually negligible. The pH value of the mixtures of sewage and sludge was generally between 7.5 and 8.0. While it may be conceded that any available method of sterilizing sewage and sludge may alter their physical and chemical properties to some extent,

Table 9. *Losses of N during the aeration of non-nitrifying mixtures of sewage and sludge, containing added ammonium sulphate and potassium nitrite, under sterile and non-sterile conditions*

The experiments were carried out in a series of flasks as detailed in Table 3 of the previous paper. In the experiment (a) the contents of the flasks were found to be sterile at the end of the experiment.

The figures refer to mg. N contained in the flasks.

Before aeration					After aeration					Loss of N
Organic- +NH ₃ -N	Added NH ₃ -N	NO ₂ -N	NO ₃ -N	Total N	Organic N	NH ₃ -N	NO ₂ -N	NO ₃ -N	Total N	
(a) Sterilized sewage and sludge mixtures with N added in the form of ammonium sulphate (5.7 mg.) and potassium nitrite (7.8 mg.): 2 days' aeration										
12.6	5.7	7.8	0.0	26.1	11.0	6.4	7.8	0.0	25.2	0.9
12.6	5.7	7.8	0.0	26.1	11.0	6.8	7.8	0.0	25.6	0.5
12.6	5.7	7.8	0.0	26.1	11.0	6.7	7.8	0.0	25.5	0.6
12.6	5.7	7.8	0.0	26.1	11.0	6.5	7.8	0.0	25.3	0.8
Total loss										2.8
N liberated as NH ₃										1.7
N presumed lost as gas										1.1
(b) Biologically active sewage and sludge mixtures with N added in the form of ammonium sulphate and potassium nitrite: 5 days' aeration										
6.4	0.0	4.2	0.0	10.6	2.8	2.5	3.1	0.0	8.4	2.2
6.4	0.0	4.2	0.0	10.6	2.8	0.7	3.0	0.0	6.5	4.1
6.4	3.2	4.2	0.0	13.8	2.4	3.7	3.4	0.0	9.5	4.3
6.4	6.3	4.2	0.0	16.9	2.6	6.2	3.2	0.0	12.0	4.9
6.4	9.5	4.2	0.0	20.1	2.8	7.6	2.5	0.0	12.9	7.2
6.4	12.6	4.2	0.0	23.2	2.8	7.6	0.8	0.0	11.2	12.0
6.4	18.9	4.2	0.0	29.5	2.3	17.4	3.1	0.0	22.8	6.7
6.4	25.2	4.2	0.0	35.8	3.0	20.0	0.4	0.0	23.4	12.4
Total loss										53.8
N liberated as NH ₃										29.0
N presumed lost as gas										24.8

there is nothing to suggest that heat sterilization would render the sewage substance less reactive chemically. There appears to be no evidence to support the belief that the losses of N reported in these papers were due to chemical reactions involving nitrite. While investigating the chemical aspects of denitrification, Fowler & Kotwal [1924] reached the conclusion that losses of N₂ in nature, in agricultural operations, or in sewage purification, were negligible as far as the chemical reactions they studied were concerned. They were unable to effect any chemical decomposition of ammonium nitrite, by maintaining solutions for 5 hr. at 50° (0.15 % NH₄NO₂ and below), and at 96° (0.014 % NH₄NO₂ and below), by the action of CO₂ or by certain other specified means.

Although we obtained slight losses of N even with sterilized sewage and sludge mixtures, in the presence of added nitrite, these losses were always very much smaller than those found with non-sterile mixtures. Larger losses were obtained during aeration of mixtures of sterilized sewage and sludge to which sterile glycine and nitrite had been added, and this raised the question as to whether the N losses in the non-sterile mixtures were due to reactions involving amino-acids. Frequent determinations during aeration experiments with sewage and sludge mixtures, however, failed to detect amino-acids in any significant amount and it is concluded that the losses of N in our experiments were not to any measurable extent associated with amino-acids but were primarily due to the action of micro-organisms, or their enzymes, upon nitrite under the conditions prescribed.

DISCUSSION

It was formerly believed that denitrification in such biological systems as sewage and soil was largely, if not entirely, an anaerobic process, although data obtained by Richards & Sawyer [1922], Reddie [1925], O'Shaughnessy & Hewitt [1935] and other investigators indicate that considerable quantities of N may be liberated during the aerobic decomposition of sewage under certain conditions.

The experiments described in these papers show that N may be lost from mixtures of sewage and sludge under a variety of conditions, and the evidence suggests that these losses occur as the result of biological activity and that they take place through the intermediate formation of nitrite. No significant losses of N were obtained in our experiments with nitrifying mixtures of sewage and sludge. The results suggest that there are many species of micro-organisms which not only possess the enzymes necessary to reduce nitrate to nitrite but are able to effect the further reduction of nitrite to N_2 . The course of the changes involved in the N cycle in experiments with pure organisms varies with the chemical nature of the environment in a manner similar to that with unsterilized mixtures of sewage and activated sludge.

The experiments described in these papers indicate that the path followed by the N depends upon two main factors; the chemical nature of the liquid phase or sewage and the nature of the enzymic mechanisms in the sludge. Activated sludges appear to contain the enzymes necessary to effect any of the changes in the following diagram:

The particular course of reactions followed varies according to the conditions which obtain, and especially as regards the proportions of available carbonaceous matter and N present and the nature of the compound in which the N occurs. The above diagram is intended to be indicative of the possible general reactions and not to show the precise route of the reactions. The reactions indicated in this diagram are encouraged by the following chemical conditions:

- (a) The presence of ammonia and assimilable carbon compounds.
- (b) Ammonia present but little or no assimilable carbon compounds.
- (c) Assimilable carbon compounds present together with nitrite and/or nitrate as the most readily available source of N.
- (d) Autolytic changes arising from absence of nutrient materials.
- (e) Fixation of N in the presence of assimilable N-free organic compounds and no readily available source of N other than free gas.

The intermediate mechanism of N fixation is still obscure, but hydroxylamine and oxime formation may be involved as suggested by Virtanen & Laine [1938].

SUMMARY

Sterilized mixtures of sewage and activated sludge inoculated with various species of micro-organisms may lose N, presumably as gas, during incubation.

This loss of N can be reduced or annulled by the addition of assimilable N-free organic compounds, such as various carbohydrates or pyruvic acid; or it may be increased by the addition of nitrate.

In addition to experiments with sterilized sewage and sludge, *Chromobacterium prodigiosum* and *Torula nitrovertens* sp. nov., a yeast isolated from sewage, and other micro-organisms have been used in experiments with a medium of known chemical composition, viz. Reader's medium. If the ammonia of this medium be replaced by nitrate a loss of N results from the growth of these organisms.

A pure culture activated sludge, prepared from *Chromobacterium prodigiosum*, reduced the B.O.D. and ammonia-N content of sterilized sewage.

It has been shown that rising of sludge, which is accompanied by nitrite formation, is encouraged by the addition of nitrite or nitrate and arrested by the presence of readily assimilable N-free organic material, such as glucose or starch.

It is suggested that while activated sludges normally contain all the requisite enzymes for the N changes discussed, the actual course followed depends upon the chemical nature of the liquid phase and the manner in which this influences the rates of the various possible enzymic reactions.

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