FORMATION OF NITRITE AND NITRATE BY ACTINOMYCETES AND FUNGI

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ABSTRACT

HIRSCH, P. (Cornell University, Ithaca, New York), L. OVERREIN, AND M. ALEXANDER. Formation of nitrite and nitrate by actinomycetes and fungi. J. Bacteriol. **82**:442–448. 1961.—Nitrite was produced by strains of *Mycobacterium*, *Nocardia*, *Streptomyces*, *Micromonospora*, and *Streptosporangium* in media containing ammonium phosphate as the sole nitrogen source. The quantity of nitrite formed was small, and the concentration was affected by pH and by the relative levels of carbon and nitrogen. *Aspergillus flavus* produced little nitrite from ammonium but formed in excess of 100 parts per million of nitrate-nitrogen. Peroxidase activity and heterotrophic nitrification were reduced in acid conditions, but mycelial development of the fungus was not markedly affected. The inability of *A. flavus* to form nitrate and nitrite at low pH appears to result from a selective effect of pH upon nitrification rather than being a consequence of the decomposition of nitrogenous intermediates.

The biological conversion of nitrogen, sulfur, phosphorus, or manganese from a reduced to a more oxidized state is not restricted to chemoautotrophic bacteria. There is abundant evidence that heterotrophic microorganisms produce substances containing nitrogen in a higher oxidation state than in the initial nitrogenous substrate. Nitrite is formed from ammonium by a variety of microorganisms that require organic carbon as energy sources; by contrast, nitrate formation from ammonium or amino compounds is restricted to only a few isolates, usually strains of aspergilli (Cutler and Mukerji, 1931; Eylar and Schmidt, 1959).

The significance of heterotrophic nitrification in terrestrial or aquatic environments is unknown. The chemoautotrophic bacteria, which have been the subject of considerable study, are usually proposed as the major if not sole agents in the biosynthesis of the nitrate found in soil and in water. There are, however, not infrequent occasions when it would seem unlikely that the small population of chemoautotrophs could account for the nitrate produced. The present investigation is designed to determine several of the factors affecting heterotrophic microorganisms active in nitrite and nitrate synthesis. Aspergillus was selected because it appears to be the most common heterotroph responsible for converting amino-nitrogen to nitrate (Eylar and Schmidt, 1959). At the same time, a survey was made of the capacity of *Actinomycetales* to accumulate nitrite since preliminary investigations indicated that these microorganisms, which are abundant in soil, could form nitrite in an ammonium medium.

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MATERIALS AND METHODS

The actinomycetes and mycobacteria were cultured in a medium containing glucose, 0.9 g; $(NH_4)_2HPO_4$, 1.65 g; K_2HPO_4, 1.4 g; KH_2PO_4, 0.8 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; NaCl, 0.01 g; $MnCl_2 \cdot 4H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.001 g; MoO_3, 0.01 g; and distilled water, 1,000 ml; final pH, 7.2. The glucose and ammonium phosphate were sterilized separately by filtration. The fungi were grown in a medium containing K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; $MnSO_4$, 0.001 g; $ZnSO_4$, 0.001 g; MoO_3, 0.001 g; distilled water, 1,000 ml; and varying amounts of $(NH_4)_2HPO_4$ and either glucose or sucrose as specified below. In studies of the effect of pH upon nitrate formation, the medium, made up at twice the usual concentration, was mixed with equal volumes of 0.07 M aconitate buffer, 0.1 M phosphate buffer, 0.1 M tris(hydroxymethyl)-aminomethane buffer, or 0.02 M borate buffer. Aconitate buffer was used below pH 6.0, phos-

phate from pH 6.0 to 7.6, tris(hydroxymethyl)-aminomethane from pH 8.0 to 9.0, and borate buffer above pH 9.0.

To obtain sufficient mycelium for studies of peroxidase activity, a medium was prepared by mixing the twofold concentrated inorganic salts solution with an equal volume of 0.1 M phosphate buffer, pH 7.0. Sucrose and $(NH_4)_2HPO_4$ were added to a final concentration of 15.0 and 6.0 g per liter, respectively. The culture was vigorously aerated by bubbling sterile air through the medium.

The organisms were grown at 30 C in Erlenmeyer flasks containing 50 or 100 ml of medium except that the mass cultures were carried out in 10-liter serum bottles. In experiments concerned with the accumulation of nitrite by actinomycetes, all glassware was washed with acid, and uninoculated controls were carried through all experimental procedures.

Aspergillus flavus strain CMC 5 was provided by E. L. Schmidt and *Nocardia autotrophica* (*Streptomyces autotrophicus*) by A. Takamiya, to whom the authors express their gratitude. The taxonomy of the nocardias is discussed by Hirsch (1960). *Aspergillus flavus* strain 59 was isolated from the Dunkirk silt loam, a New York soil. Strains of *Mycobacterium*, *Nocardia*, *Streptomyces*, *Micromonospora*, and *Streptosporangium* were either laboratory stock cultures or fresh soil isolates.

Ammonium was determined colorimetrically (Wilson and Knight, 1952), nitrite was estimated by the α-naphthylamine-sulfanilic acid procedure (Standard Methods, 1955), and total nitrogen by the Kjeldahl method (Kolthoff and Sandell, 1943). Nitrate was determined either by the reduction of nitrate to nitrite (Middleton, 1959) or by the phenoldisulfonic acid method as described by Morrill (1959). For dry weight determinations, the mycelium was washed in distilled water and dried at 85 C for 48 hr.

Peroxidase activity was assayed by a modification of the method of Heitefuss, Stahmann, and Walker (1960). Cell extracts were prepared by grinding the moist mycelium with glass beads (Minnesota Mining and Manufacturing Company, Superbrite type 100) and removing the unruptured hyphae by centrifugation at 4,000 $\times$ *g* for 15 min. Routine peroxidase assays were performed by incubating together 0.1 M phosphate buffer, 1.0 ml, pH 7.0; 0.10% hydrogen peroxide, 0.50 ml; 0.01 M pyrogallol, 1.0 ml; mycelium extract; and sufficient water to give a final volume of 5.0 ml. The pyrogallol solution was prepared immediately prior to use. The change in optical density at a wavelength of 420 mμ was determined during a 150-sec period, and the results expressed as the change in optical density per 100 μg of nitrogen in a 10-sec period, calculated from the linear part of the curve. In the study designed to determine the effect of pH on peroxidase activity, 0.02 M aconitate, phosphate, tris(hydroxymethyl)aminomethane, or borate buffers were used to replace the phosphate in the assay; the ranges in which the buffers were used are listed above.

TABLE 1. *Frequency of nitrite formation by isolates of Actinomycetales*

Microorganism	No. of strains tested	No. of strains forming nitrite		
		Rapidly	Slowly	Total
Mycobacterium sp.	2	1	0	1
Nocardia corallina	1	1	0	1
Nocardia petroleophila	7	0	1	1
Nocardia saturnea	2	1	0	1
Nocardia autotrophica	1	1	0	1
Streptomyces sp.	144	26	14	40
Micromonospora chalcea	1	1	0	1
Micromonospora sp.	2	1	0	1
Streptosporangium sp.	1	0	1	1

RESULTS

Approximately 200 strains representing five genera of *Actinomycetales* were tested for their ability to grow and produce nitrite in a glucose-ammonium-inorganic salts medium. Most of these isolates grew well in the simple medium in the absence of growth factors; further study of strains in the genera *Mycobacterium*, *Nocardia*, *Streptomyces*, and *Micromonospora*, that developed poorly or not at all in the medium was discontinued. Of those which did grow vigorously, 48 produced nitrite in detectable quantities (Table 1). A positive qualitative test at 5 days was arbitrarily chosen to indicate an active actinomycete, a positive test at 17 but not at 5 days arbitrarily selected for the designation of slow nitrite producers. The data indicate that nitrite can be formed in an ammonium medium by species of *Mycobacterium*, *Nocardia*, *Strepto-*

TABLE 2. *Nitrite production by several actinomycetes*

The values, corrected for nitrite in uninoculated control flasks, represent the mean of five replicates.

Microorganism	Nitrite formed		
	8 Days	20 Days	34 Days
	μg N/ml		
Nocardia 70	0.00	0.00	0.04
Nocardia petroleophila	0.00	0.05	0.12
Nocardia autotrophica	0.00	0.03	0.01
Streptomyces 248		0.04	0.04
Streptomyces 250	0.05	0.06	0.06
Streptomyces 254	0.03	0.02	0.01
Streptomyces 256	0.06	0.04	0.03
Streptomyces 259	0.36	0.38	0.39
Streptomyces 261		0.05	0.06
Streptomyces 269	0.05	0.05	0.04

myces, *Micromonospora*, and *Streptosporangium*. All of the more active nitrite formers, except for ten of the streptomycetes and *Nocardia autotrophica*, metabolized the nitrite further so that the concentration diminished with time.

Several of the strains exhibiting the capacity to produce nitrite in the preliminary survey were tested quantitatively. The possibility of transfer of nitrite with the inoculum was minimized by three successive washings in 0.006 M phosphate buffer, pH 7.0. The data in Table 2, representing only a portion of the results obtained, demonstrate that most strains formed only traces of the nitrogenous product, rarely more than 0.06 μg of nitrite-nitrogen per ml. However, as the colorimetric test is very sensitive, values in excess of 0.01 μg are considered significant. With many of the isolates, these trace quantities disappeared slowly, the disappearance possibly being a result of microbial utilization or nonbiological changes. Chemical decomposition of nitrous acid or a Van Slyke reaction leading to nitrite loss are unlikely since the final pH of the medium after growth of the test organisms was never below pH 6.9; nitrate may have been formed, but quantitative tests of several of the actinomycetes revealed none. Two of the strains, *Nocardia petroleophila* and Streptomyces strain 259, formed appreciable quantities of nitrite, the concentration reaching 0.16 and 0.41 μg of nitrite-nitrogen per ml by the end of the 7th week.

Streptomyces strain 259 utilized either ammonium or nitrite salts as nitrogen sources in the glucose-inorganic salts medium. For example, no nitrite was detected 5 days after inoculation of the microorganism into a medium in which the $(NH_4)_2HPO_4$ was replaced with 50 or 150 μg of nitrite-nitrogen per ml, and growth of the actinomycete was appreciable. Nitrite did not reappear,

TABLE 3. *Effect of carbon and nitrogen on nitrite accumulation*

Values are corrected for nitrite in controls and represent the mean of three replicates.

C:N ratio		24:1	10:1	3:1	1:1	1:3	1:3	1:10
Glucose, g/liter		20.6	8.80	2.64	0.88	0.29	0.88	0.88
$(NH_4)HPO_4$, g/liter		1.65	1.65	1.65	1.65	1.65	4.95	16.50
Actinomycete	Days	Nitrite-nitrogen						
		μg/N/ml						
Streptomyces 259	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	12	0.00	>1.00	0.90	0.15	0.05	0.09	0.00
	20	0.00	2.28		0.15	0.05	0.15	0.01
	28	0.00	2.39	0.96	0.15	0.03	0.16	0.04
Nocardia autotrophica	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	12	0.03	0.02	0.11	0.13	0.08	0.10	0.00
	20	0.00	0.00	0.05	0.05	0.06	0.11	0.02
	28	0.00	0.00	0.00	0.03	0.00	0.13	0.06
Nocardia petroleophila	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	12	0.15	0.15	0.16	0.16	0.11	0.00	0.00
	20	0.00		0.18	0.35	0.16	0.00	0.00
	28	0.00	0.14	0.21	0.54	0.15	0.00	0.00

presumably because of the high carbon content of the medium and the small amounts of nitrogen supplied.

The fact that no nitrite was found in solutions containing insufficient nitrogen in relation to the carbon supply suggested that the accumulation was affected by the relative quantities of the carbohydrate and nitrogen sources. To test this possibility, the concentrations of glucose and $(NH_4)_2HPO_4$ were varied to give a range of C:N ratios. Three representative actinomycetes were selected: Streptomyces strain 259, which produced the most nitrite, *Nocardia petroleophila*, which formed smaller quantities, and *Nocardia autotrophica*, an organism which did not form significant amounts of nitrite in the preliminary survey. The changes in nitrite during a 28-day period are shown in Table 3.

The most active actinomycete, Streptomyces strain 259, released nitrite only after the maximal cell yield had been attained, i.e., apparently full growth was achieved by the 3rd day in media with C:N ratios of 10:1, 3:1, and 1:1, but nitrite was not detectable until some time thereafter. In this organism, the concentration reached a maximum at about 2 to 3 weeks and remained essentially constant thereafter. Provided that the ammonium supply was not insufficient to meet the assimilatory demands of the streptomycete, as in the medium with a C:N ratio of 24:1, the amount of nitrite produced was proportional to the glucose concentration and, therefore, presumably to growth—except that the high nitrogen level (C:N of 1:10) inhibited both growth and nitrite accumulation. By contrast, *N. autotrophica* produced only small quantities of nitrite, and even these low concentrations disappeared at all but the lowest C:N ratios. The disappearance was not the result of the acid-dependent decomposition of nitrous acid because, with the exception of the treatments receiving 2.06% glucose, the pH never fell below pH 6.0.

N. petroleophila accumulated somewhat more nitrite than *N. autotrophica*. It differed further in that the nitrite persisted in the culture filtrate except when the nitrogen content of the medium was too low to meet the microorganism's assimilatory demand. The magnitude of nitrite accumulation by this actinomycete was not proportional to the initial carbohydrate level. *N. petroleophila* was particularly sensitive to the concentration of the ammonium salt, as little as 0.5% markedly suppressing growth and nitrite accumulation.

TABLE 4. *Influence of pH on ammonium oxidation by Aspergillus flavus strain CMC 5*

Incubation period of 21 days.

Initial pH	Final pH	Nitrite formed	Nitrate formed
		μg N/ml	*μg N/ml*
3.0	2.9	0.07	0.0
5.5	5.1	0.04	1.0
6.0	5.6	0.03	3.5
7.0	6.2	0.06	5.9
7.6	7.1	0.02	5.0
8.0	6.8	0.00	<1.0
9.6	8.5	0.00	0.0

The data cited above indicate that the ability to excrete nitrite in an ammonium medium is common to many actinomycetes. With *Aspergillus flavus*, on the other hand, it would appear that organic nitrogen compounds but not ammonium salts are nitrified (Eylar and Schmidt, 1959). However, microbial decomposition of organic nitrogen compounds commonly results in an increase in alkalinity whereas ammonium assimilation has the reverse effect.

In order to determine whether nitrate formation required organic nitrogen-containing substrates or whether the process was markedly pH-dependent, a medium containing 10.0 g of glucose and 10.0 g of $(NH_4)_2HPO_4$ was used. The data of Table 4 demonstrate that ammonium was indeed oxidized by *A. flavus* although the amount of nitrate formed under the experimental conditions was small. Optimum for the nitrification of ammonium was a near neutral reaction. For the fungus, nitrate was the dominant product, the nitrite concentration never being appreciable.

In view of the marked influence of acidity on nitrate formation by *A. flavus*, an investigation was made to determine whether the quantity of nitrite accumulating as a result of actinomycete development could be increased by growing the cultures at an alkaline reaction. Streptomyces strain 259 and several other *Streptomyces* and *Nocardia* isolates active and inactive in nitrite production were grown in solutions maintained at pH 6.0, 7.0, and 8.0 by means of phosphate buffers. There was noted an effect of pH on the cell yield and nitrite level for each of the test

TABLE 5. *Effect of sugar and ammonium concentration on nitrate accumulation by Aspergillus flavus strain 59*

Medium ingredients		Nitrate formed			Mycelial weight		
Sucrose	$(NH_4)_2HPO_4$	7 Days	17 Days	21 Days	7 Days	17 Days	27 Days
%		μg N/ml			mg/ml		
0.1	0.6	2.0	2.3	3.5	0.08	0.58	0.97
0.3	0.06	7.4	8.8	8.6	0.19	0.86	1.92
0.3	0.6	10.6	20.4	27.0	0.27	1.08	2.58
0.3	1.8	33.4	36.8	38.0	0.67	1.49	3.10
1.5	0.6	32.0	70.5	104.0	2.01	4.81	7.05
4.0	0.8	54.3	84.0	118.0	3.84	10.90	13.10

organisms, the nitrite concentration often increasing two- to threefold with change in hydrogen ion concentration, but none of the strains liberated appreciably higher quantities of nitrite than previously noted. For most of the actinomycetes, the amount produced was as high or higher at the neutral pH than at the other hydrogen ion concentrations; Streptomyces strain 259, however, was most active in alkaline conditions.

During the course of these studies, an isolate of *A. flavus* (strain 59) particularly active in nitrification was obtained from soil. As the relative concentrations of the carbon and nitrogen sources markedly affected the actinomycetes, a similar investigation was made of the fungus. The results summarized in Table 5, obtained with a medium buffered at pH 7.3, demonstrate that appreciable nitrate was formed by the oxidation of ammonium. Apparently the final nitrate level was influenced by both the sugar and the ammonium concentrations. For maximal nitrate production, the level of both ingredients must be high. Under proper conditions, in excess of 100 μg of nitrate-nitrogen was detected.

Kuznetzov (1950) has suggested a role for peroxidase in the nitrification reaction of heterotrophic microorganisms. In view of the great influence of acidity on nitrate accumulation by *A. flavus* strain 59, it was of interest to determine whether the little nitrate formed at acid reaction was a consequence of the elimination of an acid-sensitive peroxidase. The data of Table 6 demonstrate that, although the fungus does have a peroxidase, the enzyme was formed by fungus cultures grown at pH values at which no nitrate was detected. The greatest peroxidase activity was exhibited by mycelium grown at pH 6.5 to 7.0. By contrast, nitrate formation was the most pronounced at pH 7.0 to 7.5, similar values being noted for the optimum for nitrite accumulation. Growth, measured by dry weight, was most favored in the vicinity of pH 6.0.

Nitrite was not found in significant quantities at any pH or at any time during the period of *A. flavus* growth. Further, nitrogen balances of the cultures grown at all of the pH values from pH 3.0 to 7.5 revealed that essentially all of the nitrogen could be accounted for as nitrate-, mycelium-, or residual ammonium-nitrogen. Thus, significant quantities of intermediary compounds did not accumulate at the high hydrogen ion concentrations. The absence of nitrate at low pH thus seems to be the result of the inability of the fungus to oxidize nitrogen in its most reduced form, rather than the result of an acid-sensitive enzyme system concerned in the metabolism of a compound containing nitrogen in a partially oxidized state.

To determine the optimal pH for peroxidase activity rather than for its formation, *A. flavus* strain 59 was grown in a medium containing

TABLE 6. *Peroxidase activity and nitrate formation by Aspergillus flavus strain 59 grown at different acidities*

Incubated for 14 days. Medium contained 1.5% sucrose and 0.6% $(NH_4)_2HPO_4$.

Initial pH	Final nitrite	Final nitrate	Dry weight	Peroxidase activity*
	μg N/ml	μg N/ml	mg/ml	
3.0	0.00	0	8.19	0.030
3.5	0.00	0	8.90	0.032
4.0	0.00	0	8.34	0.031
4.5	0.00	0	9.80	0.034
5.0	0.00	7.8	9.62	0.048
5.5	0.05	18.2	9.90	0.046
6.0	0.11	25.8	12.10	0.058
6.5	0.05	51.2	11.50	0.067
7.	0.18	141.0	11.10	0.064
7.5	0.20	134.2	11.28	0.048
8.0	0.05	52.1	10.05	0.032
8.5	0.00	14.2	10.00	0.036
9.0	0.00	4.0	9.85	0.034

* Change in optical density:100 μg of cell nitrogen:10 sec.

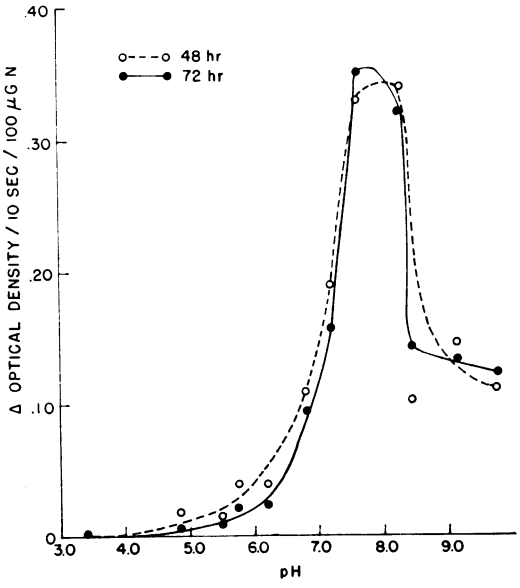

FIG. 1. *Influence of pH on the peroxidase of Aspergillus flavus strain 59. Extracts prepared from mycelium harvested at 48 and 72 hr.*

1.5% sucrose and 0.6% $(NH_4)_2HPO_4$ at an initial pH of 7.3. The mycelium was harvested at 48 and 72 hr and peroxidase assays performed on mycelial extracts. The effect of pH on the enzyme in cell extracts is shown in Fig. 1. Below pH 6.0 there was little activity, but the rate of reaction was still rapid in alkaline solution. The optimum occurred at a pH of 7.6 to 8.3. The slight enzymatic activity in acid solution is in marked contrast to the amount of peroxidase synthesized by cultures grown in acid conditions.

DISCUSSION

A variety of actinomycetes as well as an occasional mycobacterium can produce nitrite in media supplied with nitrogen in the ammonium form. This activity is apparently not restricted to the more common soil actinomycetes, that is, species of *Streptomyces* and *Nocardia*, since certain strains of *Micromonospora* and *Streptosporangium* exhibit a like capacity. Similar results have been reported for a number of actinomycetes by Eylar and Schmidt (1959) and for *Mycobacterium rubrum* by Nechaeva (1947).

None of the *Actinomycetales* reported upon herein accumulated more than 3 μg of nitrite-nitrogen per ml, and none produced detectable quantities of nitrate. Because of the sensitivity of the α-naphthylamine-sulfanilic acid method and the use of adequate replication and appropriate controls, there can be no doubt of the validity of the nitrite accumulation. However, the possibility that the nitrite arose through a biological transformation of some nitrogenous compound other than the ammonium salt supplied cannot yet be excluded. This possibility has generally been ignored in investigations of these microorganisms. For example, it is not uncommon to observe traces—of the order of 0.02 to 0.1 μg of nitrogen per ml—of a material which, on reduction, yields nitrite. This or some volatile substance rather than ammonium may be the source of part or all of the nitrite generated by the less active actinomycetes; it would probably not account for the large quantities of nitrite liberated by certain of the strains. On the other hand, a reductive rather than an oxidative mechanism is not attractive in view of the fact that (i) nitrate reduction is common to many Streptomyces and Nocardia strains, whereas only a relatively small proportion of them form detectable nitrite; (ii) activity of this type implies a synthesis of nitrate reductase after the rapid growth phase has terminated, a delayed synthesis not occurring in the nitrifying aspergilli; and (iii) nitrite appeared at a time when many actinomycetes still had available to them relatively large quantities of ammonium. But objections can be raised to all of these arguments.

In neither the fungus nor the actinomycetes was the formation of the oxidized products growth-linked. The fact that significant amounts of nitrate appear only after there are no longer increases in aspergillus cell weight may indicate that an extracellular substance or a compound released upon autolysis is acted upon by the organism. The formation, during heterotrophic growth in media containing ammonium salts, of compounds containing nitrogen in an oxidized state is not unknown. For example, Bush, Touster, and Brockman (1951) have found that *Aspergillus flavus* forms β-nitropropionic acid, and Steinberg (1939) demonstrated hydroxylamine formation during growth of *Aspergillus niger* in media supplied with ammonium, whereas *Azotobacter chroococcum* produced organically-bound hydroxylamine when cultured on ammonium-nitrogen (Saris and Virtanen, 1957).

The possession by heterotrophic nitrifiers of

peroxidase may be of importance to the nitrification sequence in these microorganisms. Little (1957) has reported that the horseradish peroxidase releases nitrite from 2-nitropropane, and there is evidence that the same enzyme will catalyze the oxidation of hydroxylamine (Cresswell and Hewitt, 1960). Should the nitrifying aspergilli be capable of forming β-nitropropionate, this may serve as the substrate for the fungal peroxidase; alternatively, the oxidation of some nitrogenous substances other than nitropropionate may be catalyzed by the enzyme.

The inability of the fungus to produce nitrate in acid conditions demonstrates a selective effect of pH on the nitrifying mechanism because the organism grows readily in acid solutions. It is possible that there is, indeed, some nitrogen oxidation but that intermediates of the reaction are decomposed to volatile products or accumulate in trace quantities. The spontaneous decomposition of nitrous acid or a reaction between nitrous acid and ammonium or amino-nitrogen could account for nitrogen losses. However, essentially all of the nitrogen supplied in the medium was recovered at the end of the incubation period as ammonium, nitrate, and mycelium-nitrogen. The only poor nitrogen recoveries occurred above pH 7.5 when ammonia volatilization undoubtedly took place.

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