Nitrification of Oxime Compounds by Heterotrophic Bacteria

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SUMMARY: Production of nitrite from the oxime of pyruvic acid was observed with three groups of heterotrophic soil bacteria: *Nocardia corallina*, *Alcaligenes* spp. and *Agrobacterium* spp. The first could nitrify up to 64 % of the oxime-nitrogen in an inorganic salts solution, the second and third up to 43 and 36 %, respectively. Glucose inhibited nitrification by stimulating the synthesis of cell substance; nitrification by *Nocardia corallina* ceased at C:N ratios above 20 :1. Approximately 75-90 % of the oxime-nitrogen was recovered as nitrite + cell-nitrogen in cultures of *Nocardia* and *Alcaligenes* spp. In peptone medium *Nocardia corallina* converted the oxime almost quantitatively to nitrite; the rate of nitrite production was parallel with that of cell multiplication. *Alcaligenes* and *Agrobacterium* spp. caused a further removal of the nitrite in peptone medium, the latter organism by denitrification. The oxime of oxaloacetic acid was readily nitrified by *Alcaligenes* spp. Acetoxime was only nitrified to a slight extent and free hydroxylamine apparently not at all. Several other organisms, of which *Corynebacterium equi* was a possible exception, did not nitrify pyruvic acid oxime.

Hydroxylamine and its aldehyde and ketone derivatives, the oximes, are known to arise in certain microbial processes. Endres (1935), Horner & Burk (1939), Aso, Migita & Ihda (1939), and Virtanen & Laine (1939) found small amounts of oxime in *Azotobacter* cultures grown with molecular nitrogen, nitrate or nitrite as source of nitrogen. Virtanen, Hakala & Järvinen (1949) reported that *Azotobacter* also forms oxime when utilizing ammonia. Virtanen & Laine (1939) found the oxime of oxaloacetic acid (oximosuccinic acid) among the products excreted from root nodules of pea plants, together with small amounts of nitrite that were probably formed by decomposition of the oxime. Hydroxylamine, either as the free compound or as an oxime, has been detected as an intermediate in the reduction of nitrate and nitrite by bacteria other than *Azotobacter* (Blom, 1928; Lindsey & Rhines, 1932; Aibel, 1938), by yeast (Virtanen & Csáky, 1948), and by fungi (Steinberg, 1939). According to Steinberg, *Aspergillus niger* also produces hydroxylamine when growing on ammonia or urea, especially the latter. The formation of hydroxylamine and oximes may thus take place by oxidation of ammonia as well as by reduction of nitrate or nitrite. The utilization of these compounds by micro-organisms has been little studied. Free hydroxylamine is known to be toxic and has generally been found not to be metabolized at low (non-toxic) concentrations. It seems to be utilized to a slight extent by *A. niger* (Steinberg, 1939) and *Torulopsis* (Virtanen & Csáky, 1948), but its tendency to decompose spontaneously into ammonia and nitrogen makes the conclusion appear uncertain. Maurer (1928) observed reduction of pyruvic acid oxime to alanine by yeast. Virtanen (1938) stated that *Rhizobium* grows well with oxaloacetic acid oxime as source of nitrogen, and that sterile pea seedlings can utilize small concentrations of this

oxime though not that of pyruvic acid. Novak & Wilson (1948) found that oxime-N was not available to *Azotobacter*, though acetaldoxime, according to Stapp & Ruschmann (1924), is toxic to this organism. Lees & Quastel (1946) showed that pyruvic acid oxime, but not hydroxylamine, is readily nitrified in soil, and Quastel & Scholefield (1949) adduced evidence that its conversion to nitrite is due to a previously unknown type of nitrifying bacterium, probably heterotrophic; isolation of such organisms was reported but no further details given. Jensen (1950) found that pure cultures of *Nitrosomonas* were unable to nitrify pyruvic acid oxime; a search for oxime-decomposing organisms has therefore been made.

METHODS

The oxime of pyruvic acid (α -oximinopropionic acid) was prepared by mixing solutions of hydroxylamine HCl and three to six (usually four) molar equivalents of sodium pyruvate at pH 7.5–8.0. The solution was sterilized by filtration (Berkefeld) and added aseptically to the autoclaved basal medium. Sterile solutions of oxime very rarely contained more than 0.1 μg . $\text{NO}_2\text{-N/ml}$. (maximum 0.25 μg ./ml.) after incubation up to 2 weeks. The usual basal medium (referred to as medium A), was a solution of the following inorganic salts in tap water (all as %, w/v): K_2HPO_4 , 0.05; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02; NaCl , 0.05; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, trace. In some cases nutrient broth or 0.5 % (w/v) Bactopeptone was used instead. Usually 2 ml. 0.025M oxime solution were added to 8 ml. of basal medium in a 50 ml. flask (final concentration, 0.005M oxime or 70 μg . oxime-N/ml.). Inoculum was given as a thin saline suspension of cells from 2-day-old agar cultures. Duplicate cultures were incubated at 25°.

Nitrite was determined colorimetrically by the Gries-Ilosvay method, as described by Snell & Snell (1945); the culture fluid was clarified with lead acetate and phosphate, or with aluminium sulphate and sodium hydroxide, when it was desired to determine nitrogen (by semi-micro Kjeldahl) in the precipitated cell substance. Glucose was determined by the micro-method of Stiles, Peterson & Fred (1926).

RESULTS

Isolation of organisms

Enrichment cultures of oxime-decomposing organisms were made by adding soil particles to medium A containing oxime, or by adding this medium to soil samples. After incubation for about 1 week, plate cultures were made on a similar medium solidified with agar. The agar medium showed a strong nitrite reaction after incubation for another week, and numerous bacterial colonies developed from which pure cultures were obtained by plating on nutrient agar. Three different kinds of bacteria were isolated which showed nitrification in quantitative tests.

Description of the oxime-nitrifying bacteria

The first organism was readily identified as *Nocardia corallina* (Bergey's *Manual*, 1948). Young cultures showed long, branching, Gram-positive rods, initially forming small but definite mycelia, later short rods and cocci, some-

times weakly acid-fast (10 sec., 5 % H_2SO_4). Abundant, pasty, pinkish growth on glucose-nutrient agar; no liquefaction of gelatin; no indole; no acid or gas from carbohydrates. Twenty-four strains isolated from an oxime-enriched clover soil from Odum Experimental Station (Jutland) all showed nitrification. But this is not a constant character of *N. corallina*; among six strains from non-enriched soils only two nitrified oxime.

The second organism had properties in agreement with the general description of *Alcaligenes* in *Bergey's Manual* (1948): small Gram-negative rods, after 3–4 days almost coccoid, mostly non-motile but somewhat motile in broth, and producing a scant and uncharacteristic whitish growth on nutrient agar. No liquefaction of gelatine; no indole; no acid or gas from carbohydrates; alkaline reaction in peptone media and in milk which otherwise shows no pronounced change. Nitrate is reduced to nitrite in medium A, and disappears rapidly from nutrient broth without any visible gas evolution. Three strains were isolated from a red loam soil from São Paulo, Brazil. These organisms seem most nearly to resemble *A. metalcaligenes* but differ by their motility in broth.

The third organism was a single strain from oxime-enriched soil (Odum Experimental Station) apparently belonging to the genus *Agrobacterium* (*Bergey's Manual*, 1948) and resembling *A. radiobacter* in most respects, but giving no characteristic growth on potato and in milk. Denitrification, with gas evolution, took place in peptone media but not in medium A; another similar culture isolated from a non-enriched soil failed to nitrify oxime.

Quantitative experiments were made with two strains of *Nocardia corallina* (K 3 and K 6), one *Alcaligenes* sp. (B 2), and the *Agrobacterium* sp. (K 1). None of these produced nitrite from ammonium sulphate (in Winogradsky's solution for *Nitrosomonas*, with or without addition of peptone), or from alanine or asparagine. Alanine (0.05 %) appeared to be inhibitory to *Alcaligenes* and *Agrobacterium*, but was an excellent source of nitrogen for *Nocardia*.

Nitrification of pyruvic acid oxime

Medium A with different pyruvate concentrations. One strain each of the three organisms were grown in medium A with 0.005M hydroxylamine + 3, 4 or 6 molar equivalents of sodium pyruvate. All three showed vigorous nitrification (Table 1). *N. corallina* converted 50–60 % of the oxime-N to nitrite, the pyruvate concentration having little effect. *Agrobacterium* behaved somewhat erratically. *Alcaligenes* showed a decreasing formation of nitrite as the C:N

Table 1. *Effect of pyruvate concentration on nitrification of pyruvic acid oxime*
Organisms grown in medium A containing 0.005M oxime

Pyruvate (M)	C:N ratio	NO ₂ -N (μg./ml.)					
		<i>Nocardia</i> (K3)		<i>Alcaligenes</i> (B2)		<i>Agrobacterium</i> (K1)	
		5 days	12 days	5 days	12 days	5 days	12 days
0.015	7.7	40	35	25	25	20	15
0.020	10.3	40	35	18	15	7.5	25
0.030	15.4	30	35	2.3	1.4	17.5	20

ratio increased, presumably because the increasing excess of available carbon permitted assimilation of more oxime-N into cell substance.

To test this point, *Alcaligenes* sp. was grown on a larger scale (25 ml. medium in 250 ml. Erlenmeyer flask). The bacterial cells were precipitated with aluminium hydroxide and nitrogen determined in the precipitate. Table 2 shows that the increase from three to four equivalents of pyruvate did result in an increased production of cell substance; no explanation was found for the still further decrease in nitrite production observed with six equivalents of pyruvate without further increase in cell-nitrogen. Estimation of residual oxime was not attempted, because this cannot be done accurately in the presence of nitrite (Csáky, 1948).

Table 2. *Production of nitrite and bacterial substance from pyruvic acid oxime by Alcaligenes sp.*

Medium A containing 0.005 M oxime and sodium pyruvate as indicated; incubation, 7 days

	Sodium pyruvate (M)		
	0.015	0.020	0.030
NO ₂ -N (μg./ml.)	30	15	2.3
N in bacterial cells (mg./25 ml.)	0.53	0.85	0.86
Oxime-N recovered as nitrite (%)	43	21	3.3
Oxime-N recovered as cell-nitrogen (%)	30	49	49.2
Oxime-N recovered total (%)	73	70	53

Influence of glucose on nitrification. The three organisms were grown in medium A containing oxime with four equivalents of pyruvate (as in all subsequent experiments) with and without 0.8 % glucose. After 10 days' incubation cell-nitrogen was determined in the precipitate formed with aluminium hydroxide, and nitrite and residual glucose in the filtrate. As shown in Table 3, glucose inhibited nitrification by stimulating cell synthesis. With *Nocardia* and *Alcaligenes* some 80–90 % of the oxime-N was recovered as nitrate + nitrogen in cell substance. The apparent inhibition of nitrification by glucose was complete with *Nocardia*, probably because this organism readily utilizes nitrite with glucose as a source of carbon.

Table 3. *Effect of glucose on production of nitrite and of bacterial substance from pyruvic acid oxime*

Organism stated in medium A with 0.005 M oxime; glucose 0.8 % (w/v) when added

	<i>Nocardia</i> (K3)		<i>Alcaligenes</i> (B2)		<i>Agrobacterium</i> (K1)	
	—	+	—	+	—	+
Addition of glucose						
NO ₂ -N (μg./ml.) after 3 days	20	0.3	8.5	7.5	7.5	0.4
NO ₂ -N (μg./ml.) after 6 days	30	0	20	6.0	12.5	1.5
NO ₂ -N (μg./ml.) after 10 days	45	0	20	7.5	25	0.4
After 10 days:						
Glucose consumed (%)		68		35		25
Oxime-N recovered						
As nitrite (%)	64	0	29	11	36	0.6
As cell-nitrogen (%)	26	90	55	72	10	42
Total (%)	90	90	84	83	46	43

N. corallina was grown in medium A (25 ml. in 250 ml. Erlenmeyer flasks) with the addition of different concentrations of glucose sterilized by filtration. The results (Table 4) show that nitrite accumulation ceased at C:N ratios above 20:1, and again some 80–90 % of the oxime-N was recovered.

Table 4. *Effect of variation of glucose concentration on production of nitrite and of cell substance from pyruvic acid oxime*

Nocardia corallina (K3) in medium A containing 0.005M oxime; incubated for 8 days

	Glucose added (%)				
	0	0.05	0.10	0.20	0.80
C:N ratio	10.3	13.1	16.0	21.7	56.0
Glucose consumed (%)	—	100	100	100	43
NO ₂ -N (μg./ml.)	40	20	14	0	0
N in bacterial cells (mg./25 ml.)	0.41	0.93	1.04	1.35	1.41
Oxime-N recovered					
As nitrite (%)	57	29	20	0	0
As cell-nitrogen (%)	24	53	71	77	81
Total (%)	81	82	91	77	81

Nitrification in complex media. *N. corallina* showed vigorous nitrification in broth and peptone media, while the action of *Alcaligenes* and *Agrobacterium* was inconstant and mostly negative, apparently because the nitrite was further transformed by these organisms in such media. A quantitative test with *Nocardia corallina* (Table 5) showed practically complete nitrification of the oxime-N in sugar-free medium after 3 days, and some inhibitory effect of glucose, presumably owing to increased cell synthesis. The experiment was repeated with a smaller inoculum and with nitrite determinations at shorter time intervals; viable counts on nutrient agar plates were also done. Fig. 1 shows a very close correlation between nitrite accumulation and bacterial multiplication; approximately 90 % of the oxime was nitrified after 4 days. Apparently the nitrite was not utilized in the presence of peptone, but accumulated in the medium.

Table 5. *Effect of glucose on nitrification of pyruvic acid oxime in peptone medium*

Nocardia corallina in 0.4 % (w/v) Bactopeptone solution containing 0.005M oxime; glucose 0.8 % (w/v) when added

Period (days)	NO ₂ -N (μg./ml.)			
	<i>N. corallina</i> , K3		<i>N. corallina</i> , K6	
	– (glucose)	+ (glucose)	– (glucose)	+ (glucose)
3	60	25	50	26
7	70	62	65	50
11	70	55	65	55

Nitrification of other oximes

Quastel & Scholefield (1949) found nitrification of oxaloacetic acid oxime, but not of acetoxime, in soil tests. An experiment (Table 6) was made with oxaloacetic acid oxime prepared from hydroxylamine and three molar equi-

valents of sodium oxaloacetate; this oxime was readily nitrified by *Alcaligenes*, but not by *Nocardia* and *Agrobacterium*.

Fig. 1. Production of nitrite and multiplication of *Nocardia corallina* (K3) in 0.4 % (w/v) peptone solution (glucose-free) + 0.005M pyruvic acid oxime. ●—●, $\text{NO}_2\text{-N}$; ○---○, viable count. Top left diagram: nitrite formation during first 36 hr. drawn to a larger scale.

Table 6. *Nitrification of oxaloacetic acid oxime*
Organism as stated in medium A containing 0.005M oxime

Period (days)	$\text{NO}_2\text{-N}$ ($\mu\text{g./ml.}$) found			
	<i>Alcaligenes</i> (B2)	<i>Nocardia</i> (K3)	<i>Agrobacterium</i> (K1)	Sterile control
5	30	0.05	0.05	0.05
10	30	0.02	(0)	0.05

Acetoxime (prepared from hydroxylamine and four or eight molar equivalents of acetone) was only slightly attacked by *Nocardia corallina* in peptone medium, where 3–5 % of the oxime-N was nitrified after 2 weeks. It seems improbable, indeed, that acetoxime would be altogether resistant to decomposition, since

this compound is quite likely to arise in nature by association between denitrifying bacteria and *Clostridium acetobutylicum*. Blom (1928), for instance, found accumulation of oxime by addition of acetone to cultures of denitrifying bacteria.

Free hydroxylamine was strongly toxic. *Alcaligenes* sp. grew feebly in peptone solution with 0.005–0.02 % hydroxylamine HCl, but no nitrate appeared. Growth of *Nocardia corallina* was suppressed at these concentrations and visibly inhibited at 0.001–0.002 % (2–4 μ g. hydroxylamine-N/ml.); the accumulation of nitrite-N did not exceed 0.4 μ g./ml. and was hardly significantly higher than in the sterile control medium. Free hydroxylamine thus appears not to be nitrified, although the evidence is not conclusive in view of the instability of hydroxylamine, particularly in the presence of nitrite.

Tests with other organisms

A number of stock cultures (*Staphylococcus aureus*, *Bacillus subtilis*, *B. mycoides*, *Escherichia coli*, *Aerobacter aerogenes*, *Alcaligenes faecalis*, *A. metalcaligenes*, *Pseudomonas aeruginosa*, *Rhizobium* spp., *Agrobacterium radiobacter*, *Azotobacterium chroococcum*, *Mycobacterium phlei*, and various actinomycetes) failed to produce nitrite from pyruvic acid oxime in nutrient broth or in medium A. Only a strain of *Corynebacterium equi*, from the State Veterinary Serum Laboratory, Copenhagen, gave evidence of slight nitrification in peptone solution, and this did not exceed 3 % of the oxime-N after 2 weeks' incubation.

DISCUSSION

The experiments show clearly that several types of bacteria can decompose oximes with formation of nitrite and can utilize oxime-N for cell synthesis to an extent which largely depends on the supply of available carbon. Nitrite formation is probably not an energy-yielding process like ammonia oxidation by *Nitrosomonas*, but may tentatively be regarded as a hydrolytic process schematically expressed thus: $R \cdot C(:NOH) + H_2O \rightarrow R \cdot CH_2 + HNO_2$. The nitrite found in the culture medium may thus represent unutilized nitrogen accumulating as a waste product when the organic radical serves as source of energy. In agreement with this view, *Nocardia* and *Alcaligenes* (but not *Agrobacterium*) were able to grow in medium A with sodium propionate and sodium nitrate as sources of carbon and nitrogen.

On the basis of these results it is possible to agree with the suggestion of Quastel & Scholefield (1949) that 'the process of nitrification may not be wholly accomplished by the autotrophic organisms'. Since hydroxylamine can definitely be formed by denitrification and other processes of nitrate and nitrite reduction, the occasional presence of oximes under natural conditions is quite likely. In such cases the oxime-decomposing bacteria might bring about a re-nitrification of nitrogen previously reduced from the nitrate or nitrite stage. A heterotrophic nitrification of oximes not resulting from nitrate reduction might take place in the rhizosphere of leguminous plants if nitrogenous compounds are excreted from the nodules (Virtanen, 1938). Such excretion,

however, appears to be somewhat exceptional, and oxime-N rarely accounts for more than 1–2 % of the total excreted nitrogen (Virtanen & Laine, 1939). Also the quantities of oxime produced by other micro-organisms (e.g. *Azotobacter*) or by lower animals (Yamafuji, 1950) appear to be small. It thus appears doubtful whether the amounts of nitrite arising by this route would be quantitatively important in comparison with those produced from ammonia by *Nitrosomonas*. And in any case no organism other than the autotrophic *Nitrobacter* is yet known to accomplish the final stage of the whole nitrification process in soil, i.e. the oxidation of nitrite to nitrate.

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