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The biodegradation of piperazine and structurally-related linear and cyclic amines

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Abstract

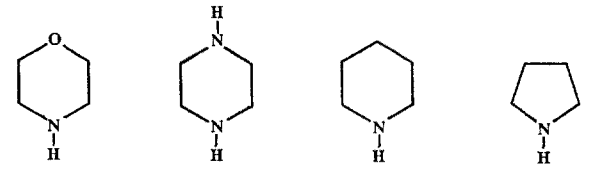
The biodegradability of a range of linear and cyclic amines was assessed. All proved to be biodegradable but there were interesting differences in their susceptibility. The least degradable was piperazine although piperazine-degrading microorganisms were of widespread occurrence in samples of water and activated sludge and, to a lesser extent, soils. Piperazine degraders are only present in very small numbers – on average *ca.* 0.8/ml of river water. Of six isolates capable of using piperazine as a sole source of carbon, nitrogen and energy in pure culture five were identified as *Mycobacterium* spp. and one as *Arthrobacter* sp., all strains were capable only of slow growth (mean generation time of *ca.* 30 to 40 hours) on this substrate. Piperidine, pyrrolidine, ethanolamine and diethanolamine were all readily biodegradable. The relationship between structure and degradability of amines is discussed as are the possible reasons for the relative recalcitrance of piperazine.

Introduction

The aliphatic amines, an important group of industrial chemicals, are produced annually in very large amounts and employed in a wide range of applications (Edens & Lochary 1992; Mjos 1978; National Research Council 1982; Turcotte & Johnson 1992). Amongst other things they are directly used as antioxidants, anti-corrosive agents, solvents, emulsifiers and drugs and are commonly found as moieties in rubber additives, pesticides, drugs and optical brighteners. Consequently aliphatic amines are found in many liquid effluents, primarily industrial but also domestic. An understanding of the biodegradation of amines is therefore of importance.

While some amines are readily biodegradable, others (often structurally similar) are not (Rothkopf & Bartha 1984; Pitter & Chudoba 1990). The biodegradation of secondary amines is particularly important due to their propensity for conversion either chemically (e.g. Mirvish 1975) or microbiologically (e.g. Calmels et al. 1985) to N-nitrosamines many of which are potent carcinogens (e.g. Bartsch 1991). The biodegradation of some amines, e.g. morpholine (Brown & Knapp 1990; Cech et al. 1988; Dmitrenko et al. 1985, 1987; Knapp & Whytell 1990; Knapp et al. 1982; Swain et al. 1991) and ethanolamine (e.g. Jones & Turner 1973; Jones et al. 1973; Roof & Roth 1988; Scarlett & Turner 1976) has been well researched. Others however have received little or no attention.

Previous research on morpholine has not yet answered the question of why this structurally simple compound (Fig. 1) is relatively difficult to degrade and why it is in general only degraded by a very limited range of bacteria (see previously cited references). There are few (Rothkopf & Bartha (1984) being a welcome exception) comparative studies of amine biodegradation to help answer this question. Piperazine is similar to morpholine (Fig. 1), its biodegradation is therefore of interest, but has attracted little attention with only one report of biodegradation (Dmitrenko et al. 1987). Commercially, piperazine is the second most important cyclic amine (Mjos 1978) and, amongst other uses, its salts are widely employed as antihelminthic drugs in human and veterinary medicine. In this paper we report on the biodegradability of piperazine and



Morpholine Piperazine Piperidine Pyrrolidine

$NH_2CH_2CH_2OH$ Ethanolamine

$HOCH_2CH_2NHCH_2CH_2OH$ Diethanolamine

$NH_2CH_2CH_2NHCH_2CH_2OH$ 2-(2-aminoethylamino)ethanol

$NH_2CH_2CH_2OCH_2CH_2OH$ 2-(2-aminoethoxy)ethanol

Fig. 1. The structure of the amines studied.

compare it with that of a range of cyclic and linear amines (Fig. 1) which have common structural features.

Materials and methods

Materials

Unless otherwise stated all chemicals were obtained from B.D.H. Ltd. (Poole, Dorset, U.K.) and were of Analar or the next purest grade available. Piperazine, piperidine, pyrrolidine, diethanolamine, 2-(2-aminoethylamino)ethanol and 2-(2-aminoethoxy)ethanol were from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, U.K.). Nutrient agar and nutrient broth were from Oxoid Ltd. (Basingstoke, Hants, U.K.) and Noble agar was from Difco (East Molesy, Surrey, U.K.). A variety of materials were examined for the presence of amine-degrading microorganisms, all were collected aseptically in sterile containers.

All media were sterilised by autoclaving at 121°C for 15 minutes.

Analysis

Amines were assayed spectrophotometrically (separate calibrations were carried out for each amine), the methods used are not specific but in all experiments the amount of interfering material in environmental samples was shown to be insignificant. Cyclic secondary amines were all assayed by an adaptation (Knapp et al. 1982) of the method of Stevens & Skov (1965). Linear amines and alkanolamines were assayed by an adaptation of the method of Dubin (1959) in which dimethyl formamide was used in place of dioxane. Ammonia was assayed using Nessler's reagent.

Die-away tests

The capability of microbes in environmental samples to degrade the tested amines was determined using die-away tests. 25 ml of water, activated sludge or soil suspensions were added to 50 ml of a sterile solution of the appropriate amine (as the sole source of carbon, nitrogen and energy)/mineral salts medium pH 7.0 (g/l distilled water:KH_2PO_4 – 2; $MgSO_4 \cdot 7H_2O$ - 0.04; $FeCl_3 \cdot 6H_2O$ - 0.004. Iron and magnesium salts were added to the medium after autoclaving separately) and a further 25 ml of sterile distilled water was added to give a final amine concentration of 1 mM. When soil was used 40 g fresh weight was agitated with 200 ml of sterile distilled water, after overnight settling 25 ml of the supernatant fluid was used as an inoculum. After inoculation cultures were incubated at 27°C with shaking (100 rpm) and samples were removed at regular intervals centrifuged for 5 min. (MSE microcentaur – full speed) and the supernatent fluid assayed for amines. Control flasks contained 20 mg $HgCl_2$ as a biostat. Under the conditions employed all amines remained in aqueous solution and did not adsorb to or partition into solids to any significant extent.

Enumeration of microbial populations and isolaton of amine-degrading bacteria

Prior to counting samples received mild ultrasonic treatment (2 minutes in an ultrasonic water bath) to break up bacterial aggregates and were then serially diluted using sodium tripolyphosphate (5 mg/l) solution. The numbers of microbes capable of degrading cyclic secondary amines was determined by Most Probable Number (MPN) counts. The method used was that described for morpholine-degraders by Knapp & Brown (1988) and depends on demonstrating removal of amines from the medium using a spot chemical test. A positive reaction is shown by the absence of amine as indicated by the lack of a colour reaction. Interference by environmental chemicals was negligible, as shown by controls lacking amine. Counts were incubated for

8 weeks for piperazine and 4 weeks for all other compounds. The numbers of organisms degrading linear amines was assessed by spread plate viable counts. Solid media contained amine at 10 mM as sole source of carbon, nitrogen and energy together with mineral salts (made up as in the die-away media) and were adjusted to pH 7.0, they were solidified with Noble agar (1.5 % w/v).

After successful die-away tests, amine-degrading cultures were developed by repeated subculture in enrichment media with amines as sole carbon and nitrogen sources. Cultures were then spread onto plates of the same media to allow isolation of amine-degrading bacteria. Relatively large colonies possibly capable of amine degradation were rigorously purified by repeated subculture alternately on amine/mineral salts agar and nutrient agar. Once pure, cultures were tested for growth and amine degradation in liquid minimal media with the appropriate amine as sole source of carbon and energy, this is essential as non-amine degrading oligotrophs can give good growth on mineral salts agar plates (Knapp & Brown 1988).

Determination of growth rate

Mean generation time was calculated from turbidimetric data by conventional methods or from data on substrate use. With the latter method $\log_{10}$ of the amount of substrate *used* was plotted against time – the amount of growth being assumed to be proportional to the amount of biomass. This gives a linear plot equivalent to a plot of $\log_{10}$ of turbidity vs. time.

Toxicity studies

The possible inhibitory effects of various amines on Gram-negative bacteria was assessed by studying the effect of the amines on the rate and extent of growth of two pseudomonads (*Ps. aeruginosa* PAO1 and *Ps. putida* KT2440). The test organisms were grown on Succinate (10 mM)/mineral salts medium (pH 7.0) (g/l of distilled water: -KH_2PO_4 -2; $(NH_4)_2SO_4$ -1; $MgSO_4.7H_2O$ -0.04; $FeCl_3.6H_2O$ -0.004) to late logarithmic phase and then inoculated into the same medium containing various concentrations of the tested compound at 1 to 100 mM. Cultures were incubated with shaking (100 rpm) at 27°C in an orbital incubator, samples were removed at regular intervals and bacterial growth was determined turbidimetrically by measurement of optical density at 560 nm.

Results

Die-away studies

The biodegradability of piperazine was demonstrated in die-away tests. Materials from a wide variety of sources were shown (Table 1) to contain piperazine-degrading microorganisms. In controls containing $HgCl_2$ no degradation occurred indicating that piperazine removal was due to biological rather than physico-chemical agency. Lag periods before degradation could be observed were long, always in excess of 14 days and in two thirds of cases greater than 26 days. A considerable time (21 to 68 days) elapsed before complete degradation and with some inocula no degradation occurred within 3 months. Piperazine degradation was quicker with activated sludge inocula than with those from water; soils were the worst source of piperazine-degrading microbes. The period between the apparent onset of degradation and its completion varied (Tables 1 & 2) but was generally in the range of 7 to 16 days.

Comparative studies of the biodegradability of selected amines were conducted using water from the River Aire in central Leeds taken in different seasons. At the sampling point this river is classed as grade 3/4, and is recovering from serious pollution by treated domestic and industrial effluents from a large conurbation. All eight amines were biodegradable (Table 2), but piperazine was the most recalcitrant. Lag periods ranged from 30 to 60 days and times for complete degradation from 40 to 68 days. Pyrrolidine and piperidine were always rapidly degraded with little or no lag period. Morpholine was also degraded faster than piperazine (4 to 10 day lag and 10 to 14 days complete degradation). Ethanolamine degradation was very rapid and while that of diethanolamine was less so, lag periods were only 1 day and degradation was complete in 4 to 5 days. 2-(2-Aminoethylamino)ethanol and 2-(2-aminoethoxy)ethanol were both degradable. The lag periods and complete degradation times for these compounds were variable, but generally similar to those observed for morpholine.

Enumeration of amine-degrading microbes

Counts of the number of amine-degrading microorganisms in River Aire water (Table 2) revealed no obvious seasonal trends. Bacteria degrading cyclic amines were counted by the MPN method and alkanolamine-degraders were counted by plate counts on selec-

Table 1. Biodegradation of piperazine in activated sludge, river water and soil.

Sample		Time for complete degradation days	Lag period prior to apparent degradation days
Waters			
1	Fairburn Ings (Lake)	48	36
2	Aire & Calder Canal (Knottingley)	61	47
3	River Aire, Knostrop, Leeds	47	31
4	Stream Nr. Birkin	53	18
5	River Aire (Beal Weir)	43	30
6	River Calder Dewsbury	39	26
	Pit tip and dump		
7	Lechate Water (Dewsbury)	No biodegradation in 3 months	
Materials from sewage works			
8	Activated sludge – Dewsbury	21	14
9	Activated sludge – Knostrop	26	16
10	Activated sludge – Owlwood	21	14
11	Humus tanks, Owlwood	53	39
Soils			
12	Stable compost (Pudsey)	24	15
13	Stream mud – Pudsey Beck	38	28
14	Garden Soil (Pudsey)	42	30
15	Garden Soil (J.S. Knapp)	68	60
16	Meadow soil, molehill (Pudsey)	65	58
17	Sykes Wood, Leaf Litter		
	Troydale Leaf Litter	No biodegradation in 3 months	
	Compost		

Water Samples 2, 3, 5, 6 and 7 are from polluted sites which are likely to have been exposed to amines in industrial or domestic effluent.
Sites 1 and 4 are not polluted.
All materials (8 to 10) from sewage works will have encountered amines in industrial or domestic effluents.
Soils from sites 12 and 13 may have been exposed to pollution, those from other sites are unlikely to have been exposed.

tive media, as these materials could not be used in the MPN test. Clearly plate counts cannot be directly compared with MPN counts. Counts on agar plates with 2-(2-aminoethylamino)ethanol or 2-(2-aminoethoxy)ethanol as sole carbon and energy source were less than those on control mineral salts agar (even when 1 mM amine was used), suggesting inhibition and making plate counts impossible. Ethanolamine- and diethanolamine-degraders were present in similar, quite large (and fairly constant) numbers in all samples. MPNs of piperidine- and pyrrolidine-degraders were generally similar and (within the limits of accuracy of the MPN technique) remained fairly constant in all samples, as did those of morpholine- and piperazine-degraders (although at a much lower level). The average MPN of morpholine- and piperazine-degraders was only *ca.* 1.5 and 0.1% respectively of that of piperidine- and pyrrolidine-degraders. Piperazine-degraders were sometimes undetectable in MPN counts. In Dewsbury and Owlwood activated sludges the MPN of

Table 2. Number of amine-degraders and times taken for microbial biodegradation of linear and cyclic amines in water taken from the River Aire in central Leeds.

Substrate	Number of amine degrading microorganisms per ml of river water			Complete degradation time time – days		Lag period – days	
	Mean[1] (± standard deviation)	Method[3]	Range	Mean[4] Value	Range	Mean Value	Range
piperidine	442 (± 281)	+	130 to 900	3	2 to 4	0.4	0 to 1
pyrrolidine	427 (± 381)	+	35 to 900	2.2	2 to 3	0.2	0 to 1
piperazine	0.37 (± 0.4)	+	0 to 0.8	54	40 to 68	44	30 to 60
morpholine	6.6 (± 5.6)	+	1.4 to 13	14	10 to 18	6	4 to 9
ethanolamine	9.86×10^4 ($\pm 10 \times 10^4$)	*	2.5 to 34×10^4	1.5	1 to 2	0	–
diethanolamine	8.52×10^4 ($\pm 8.68 \times 10^4$)	*	2.1 to 23×10^4	4.5	4 to 5	1	1
2-(2-aminoethyl-amino) ethanol	NT[2]		NT	20	8 to 30	6.5	3 to 12
2-(2-aminoethoxy) ethanol	NT		NT	21	14 to 28	8	3 to 16

[1] Numbers of amine-degraders are the mean of 5 or 6 determinations.
[2] NT = not tested.
[3] + = most probable number method used.
[3] * = plate count used
[4] Degradation times are the means of 4 or 5 determinations.

piperazine-degraders was 11 and 14/ml respectively. Piperidine- and pyrrolidine-degraders were present at> 1.8×10^5/ml in both sludges.

Isolation and identification of amine-degrading bacteria

Six different pure cultures were obtained which grew on piperazine as a sole source of carbon, nitrogen and energy, all were Gram-positive rods. Results of preliminary identification tests are given in Table 3. Five of these bacteria (Z91, Z92, Z2, Z12 & ZJY1) are acid fast rods which suggests they are members of the genus *Mycobacterium.* The sixth strain (Z8c) is not acid fast, cannot grow anaerobically and is markedly pleiomorphic with a rod – coccus cycle, suggesting that it is probably a member of the genus *Arthrobacter.* The only morpholine degrading strain isolated also appeared to be *Mycobacterium* spp.

All piperazine degrading strains grew well in 10 mM piperazine-mineral salts medium converting most (from 75 to *ca.* 100%) of the amine-nitrogen to ammonia by stationary phase. Interestingly the strains isolated on piperazine did not use morpholine as a growth substrate and the morpholine-degrading strain did not grow on piperazine. Both piperazine- and morpholine-degrading strains could use piperidine and pyrrolidine for growth.

The only bacteria able to grow on 2-(2-aminoethylamino)ethanol (aE4 and aE2) or on 2-(2-aminoethoxy)ethanol (OX3) were immotile, non-sporing, non-acid fast, Gram positive rods (detailed identification was not attempted). A wide range of bacteria capable of growth on piperidine and pyrrolidine were isolated, all were rapidly growing Gram negative rods – several strains were tentatively identified as *Pseudomonas* spp. All organisms isolated as piperidine-degraders were found to degrade pyrrolidine and *vice versa.* The diethanolamine-degraders isolated were mostly Gram negative, while a mixture of Gram positive and negative bacteria were isolated on ethanolamine.

Growth rates

The relative rates of growth on minimal and complex media were readily apparent from visual comparison of cultures on solid media. Many of the organisms isolated on the alkanolamines, piperidine and pyrrolidine were intrinsically fast growing, even on minimal media, while those isolated on piperazine and morpholine were not. This was further demonstrated when selected typical isolates were cultured on minimal media with an amine as their sole carbon and energy source. While most piperidine, pyrrolidine, diethanolamine and ethanolamine-degraders are

Table 3. Preliminary identification tests on piperazine-degrading bacteria.

Strain	A-F	Shape	37	Colour	Colony	Liquid culture
Z12	+	rod	–	yellow	glistening	turbid
ZJY_1	+	rod	NT	yellow	glistening	turbid
Z92	+	rod	+	white	dry waxy	greasy film turbid & clumps
Z91	+	rod	NT	white	dry waxy	greasy film turbid & clumps
Z2	+	rod	+	white	dry waxy	clumps
Z8c	–	pleio	–	white	mucoid	turbid

A-F = Acid fast.
37 = growth at 37°C.
Colony = colonial appearance.
Liquid = style of growth of liquid cultures.
Pleio = pleiomorphic – showing a pronounced rod/coccus cycle.
NT = not tested.

All strains were Gram-positive, immotile, non-sporing and showed no trace of mycelial development.They were also Catalase positive, oxidase negative and were oxidative in the Hugh and Leifson O/F test.

Table 4. Generation time of amine-degrading isolates utilising the substrates on which they were isolated as sole sources of carbon, nitrogen and energy.

Strain	Gram reaction	Generation time - Hours	Generation time Method[1]	Media
Pip 1	-	2.3	x	piperidine (10 mM)
Pip 9	-	1.2	x	piperidine (10 mM)
dine 1	-	2.6	x	piperidine (10 mM)
pyro 2	-	1.5	x	pyrrolidine (10 mM)
pyro 3	-	2.8	x	pyrrolidine (10 mM)
pyro 8	-	1.2	x	pyrrolidine (10 mM)
Di2	-	2.3	x	diethanolamine (10 mM)
Di3	+	9	x	diethanolamine (10 mM)
Di1	-	18	x	diethanolamine (10 mM)
E1	+	4	x	ethanolamine (10 mM)
E2	+	3	x	ethanolamine (10 mM)
Mor	+	14	xx	morpholine (10 mM)
Z91	+	34	xx	piperazine (10 mM)
Z2	+	37	xx	piperazine (10 mM)
Z12	+	38	xx	piperazine (10 mM)
Z92	+	31	xx	piperazine (10 mM)
Z8C	+	29	xx	piperazine (10 mM)

[1] x = data were obtained turbidimetrically (560 nm).
[1] xx = data were obtained by die-away.

Table 5. Generation time of six strains of piperazine degrading bacteria on different media.

		Media[1]						
		G + T80	A + T80	N.B + T80	Z + T80	T80	(Z + T80)	Z
	Acid	Turbidimetrically					Die-away	
Strain	fast reaction							
Z2	+	14	12	11	7.5	12	9	37
Z8C	-	8	NG	7	20	NG	26	29
Z12	+	12	8	10	13	13	17	38
ZJY1	+	13	16	8	17	NT	18	NT
Z91	+	NT	11	12	10	NT	14	34
Z92	+	NT	10	9	13	9	17	31

[1] G + T80 = Glucose (5 mM) + 1% Tween 80.
A + T80 = Acetate (20 mM) + 1% Tween 80.
N.B + T80 = Nutrient broth + 1% Tween 80.
Z + T80 = Piperazine (10 mM) + 1% Tween 80.
Z = Piperazine (10 mM) alone.
T80 = Tween 80 (1%) alone.
NT = Not tested.
NG = No growth.

All media were at pH 7.0 and contained mineral salts, media G + T80, A + T80 and T80 all contained $(NH_4)_2SO_4$ (1 g/l) – other media did not.

Table 6. Effect of 2-(2-aminoethylamino)ethanol and 2-(2-aminoethoxy)ethanol on the growth of two *Pseudomonas* spp.

	Pseudomonas aeruginosa		*Pseudomonas putida*	
Medium[1]	μ (hr^{-1})[2]	M.G.[3]	μ (hr^{-1})	M.G.
S	0.58	0.75	0.41	0.75
S + aE 10 mM	0.35	0.4	0.35	0.45
S + aE 100 mM	0.13	0.18	0.30	0.36
S + OX 10 mM	0.35	0.3	0.25	0.45
S + OX 100 mM	0	0	0.23	0.43

[1] S = Succinate (10 mM)/mineral salts,
aE = 2-(2-aminoethylamino)ethanol,
OX = 2-(2-aminoethoxy)ethanol.
[2] μ = specific growth rate (hr^{-1}).
[3] M.G. = Maximum growth (A_{560nm}) in 8 hours.

capable of rapid growth (generation times < 4 hours), morpholine- and even more so piperazine-degraders were only capable of slow growth (Table 4).

In liquid media piperazine-degrading bacteria (except Z12 and Z8c) did not show homogeneous growth but grew solely or largely in the form of small, waxy clumps about 1 mm in diameter or partly as greasy films and clumps with some turbid growth (Table 3). Growth could not therefore be monitored turbidimetrically. Addition of Tween 80 (1 % w/v) to liquid media resulted in much more homogeneous growth and allowed turbidimetric assay. Growth rate in clumping or greasy cultures was measured using a method in which the rate of change of substrate utilisation with time was assessed. The generation times of piperazine-degraders were measured in several media

(Table 5). Growth on a rich medium (e.g. nutrient broth) was little faster (and sometimes slower) than on minimal media thus these organisms are intrinsically slow growing. For most organisms the growth rate on piperazine plus Tween (whichever method was used to measure it) was similar to, or only a little slower than, that on other media. Z8c however grew much (*ca.* 60 %) slower on piperazine than on other media and could not utilise Tween 80. Mean generation times calculated from the rate of substrate utilisation showed fairly close agreement with those derived from turbidimetric data. However growth of the mycobacteria (Z2, Z12, Z91 and Z92) on piperazine alone was much slower (about half the rate) than in the presence of Tween 80. Comparison with the growth rates obtained on Tween 80 alone suggests that in media containing piperazine plus Tween 80 the rate of mycobacterial growth depends on use of Tween 80 as the principle energy source, however utilisation of piperazine is not repressed by utilisation of Tween 80.

Toxicity of amines

The limited range of bacterial types (all Gram positive) that could degrade piperazine, morpholine, 2-(2-aminoethylamino)ethanol and 2-(2-aminoethoxy)ethanol, and also the problems in enumerating organisms capable of growth on the latter two compounds, suggest that some of these amines may be inhibitory to bacterial growth. Accordingly the effect of these compounds on the growth of two typical pseudomonads on minimal media was assessed. At up to 100 mM piperazine and morpholine (and also ethanolamine, diethanolamine, piperidine and pyrrolidine) did not affect the rate or extent of growth of these organisms. 2-(2-aminoethylamino)ethanol and 2-(2-aminoethoxy)ethanol however were inhibitory to both organisms, reducing the rate and extent of growth compared to controls (Table 6). Nevertheless at 10 mM neither of these compounds caused more than a 40% decrease in the rate of growth of the test organisms and when the organisms were grown on nutrient broth neither compound appeared to be inhibitory.

Discussion

This study confirms the brief report by Dmitrenko et al. (1987) that piperazine is biodegradable but supports their contention that it is relatively resistant to degradation. We have shown piperazine-degrading bacteria to be fairly widespread in surface waters and activated sludge but not so regularly encountered in soils. Piperazine was by far the most recalcitrant of the amines studied – the average time for its complete degradation was more than 10 times that for degradation of ethanolamine, diethanolamine, piperidine and pyrrolidine, *ca.* 3 times that for morpholine and *ca.* 2.5 times that for 2-(2-aminoethylamino)ethanol or 2-(2-aminoethoxy)ethanol.

The rapid degradation of the ethanolamines, piperidine and pyrrolidine agrees with previous findings (Agarwal et al. 1988; Gupta et al. 1975; Rothkopf & Bartha 1984). The ready degradability of ethanolamine is not surprising since, as a common biological product (constituent of phospholipids), it is likely that many organisms will be adapted to degrade it. Diethanolamine, probably not a biological product, is a common industrial chemical (Anon. 1983) and the existence of large numbers of bacteria capable of its degradation is not unexpected. Although we isolated several diethanolamine degrading bacteria in this study, degradation of this compound was not studied in detail and there appear to have been few such studies (Williams & Callely 1982; Gannon et al. 1978). The rapid degradation of both ethanolamines and of piperidine and pyrrolidine is no doubt accounted for by the relatively high numbers of organisms (some rapidly growing) which are capable of growth on these substrates.

The biodegradation of 2-(2-aminoethylamino)ethanol and 2-(2-aminoethoxy)ethanol has not previously been reported. 2-(2-Aminoethylamino)ethanol is very similar to diethanolamine in structure and it might have been expected that the two would be degraded by the same or similar organisms. This was clearly not the case as both the isolates degrading the former were Gram-positive, non acid-fast rods, while the majority of those degrading the later were Gram-negative rods.

The numbers of morpholine-degrading microbes and the times taken for morpholine degradation in die-away tests are similar to those previously reported (Knapp & Whytell 1990). The only morpholine-degrader isolated in this study was a *Mycobacterium*, this is not surprising as in most previous studies (Brown 1988; Brown & Knapp 1990; Cech et al. 1988; Dmitrenko & Gvozdyak 1988; Knapp et al. 1982; Knapp & Brown 1988; Knapp & Whytell 1990) the only isolates utilising this substrate (as sole carbon, nitrogen and energy source) were mycobacteria. The one exception being the report (Dmitrenko et al. 1985) of the isolation of an *Arthrobacter* which could grow on

morpholine and also a variety of other Gram-positive bacteria that could co-metabolise it. The growth rate of the mycobacterium isolated here was similar to those reported by Brown & Knapp (1990) and Cech et al. (1988).

Of the piperazine-degrading bacterial strains isolated five were *Mycobacterium* spp. and one an *Arthrobacter.* The only bacterium previously reported (Dmitrenko et al. 1987) to utilise this substrate was also an *Arthrobacter.* Thus all bacteria so far reported to use morpholine or piperazine as sole source of carbon, nitrogen and energy belong to just two genera. However with other similar substrates the range of degradative organisms is not so narrow.

What feature of these two substrates is it that allows degradation only by a narrow range of bacteria, which are all slow growing? Morpholine and piperazine are not inhibitory to the growth of rapidly growing pseudomonads so often encountered as biodegraders of xenobiotic compounds, including amines. Therefore selective toxicity of these compounds cannot provide an explanation. Clearly it is not the heterocyclic nature of the compounds or steric factors (e.g. shape and size) as the ability of pyrrolidine and piperidine to support growth of rapidly growing Gram-negative bacteria has been demonstrated and growth of *Pseudomonas* spp. on pyrrolidine (Jakoby & Fredericks 1959) and piperidine (Agarwal et al. 1988) has been reported. Morpholine and piperazine are composed of two 2-carbon (C_2) units. However the ability to utilise C_2 units cannot restrict use of these substrates since this study and others referred to earlier have shown this ability is very widespread among microorganisms. Aerobic degradation of morpholine and piperazine probably results in production of highly oxidised C_2 units, however it is unlikely that the ability to utilise these intermediates is the selective factor. Compounds like nitrilotriacetic acid have been shown to be degraded by pseudomonads (e.g. Cripps & Noble 1973) via such intermediates as glycine and glyoxalate, and even oxalic acid is also degraded by similar organisms (Kornberg 1966). The most likely explanation is that the simultaneous presence in the heterocyclic ring of two very electronegative elements makes the initial degradative steps, of C-N or C-0 bond cleavage, more difficult due to their electron-withdrawing effects. The electron distribution within the heterocyclic ring may mean that these substrates can only be efficiently degraded by a restricted range of enzymes when compared to the more usual secondary aliphatic amines. Most evidence to date suggests that the initial attack on secondary amines by aerobic bacteria or yeasts (e.g. Large 1971; Fattakhova et al. 1991; or other papers cited above) is by oxidative mechanisms. Comparison of the catalytic properties of amine-oxidising enzymes from *Mycobacterium* or *Arthrobacter* spp. with those from Gram-negative bacteria e.g. *Pseudomonas* would be very valuable.

Interestingly growth of the mycobacterial piperazine degraders is much slower in the absence of Tween 80 than in its presence. This could be related to the more homogeneous growth (and better mass transfer) engendered by Tween 80, however this seems unlikely as strain Z12 grows homogeneously even in the absence of Tween 80. Brown (1988) showed that strains of morpholine-degrading mycobacteria including ones that clumped in liquid media grew at similar rates irrespective of the presence of Tween 80. All the mycobacteria in our study could utilise Tween 80, thus the greater growth rates observed in the presence of Tween were probably due to its use as a substrate. If this is so the rates of growth on piperazine are particularly low, suggesting either the intrinsic resistance of piperazine to degradation or possibly a problem with its ability to enter the bacterial cell.

It is unclear why piperazine is less amenable than morpholine to biodegradation although both our study and that of Dmitrenko et al. (1987) have found this to be so. The slower degradation in die-away tests may be accounted for partly by the smaller numbers of piperazine degraders as compared to morpholine degraders found in the aquatic environment. These small numbers may be due to the low degree of environmental contamination with piperazine which is mainly used as a veterinary drug, whereas morpholine is a major industrial and commercial chemical. Previous studies (e.g. Fushiwaki & Urano 1988; Knapp & Whytell 1990; McKenzie & Hughes 1976) suggest the ability of aquatic microflora to degrade a synthetic chemical may be related to the degree of pollution of the sampling site.

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