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Biodegradation of *s*-triazine xenobiotics

Alasdair M. Cook

Department of Microbiology, Swiss Federal Institute of Technology, Zürich, Switzerland

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1. SUMMARY

Biodegradation of xenobiotic compounds is examined with *s*-triazines as an example and with biological treatment of wastewater containing *s*-triazines as an aim. *s*-Triazines have been termed recalcitrant, but examination of the literature indicates that a potential for biodegradation exists. Nitrogen-limited enrichment cultures yield organisms able to degrade by-products of the industrial synthesis of *s*-triazine herbicides as sources of nitrogen. The choice of inoculum for these enrichments is important and often allows for successful enrichment after simple batch culture, but organisms containing several degradative reactions could be obtained only after selection in extended culture. Routine, specific determinations of all *s*-triazines (by HPLC) were essential throughout the work. Molar growth yields show complete mass balances for the utilization of *s*-triazines. Kinetic experiments indicate that specific degradation rates of *s*-triazines in growing cells are about 0.4 mkat/kg of protein. Characterised biochemical pathways consist of a series of hydrolytic cleavages of chloro-, amino- and alkylamino-groups from the *s*-triazine ring. Pathways converge to cyanuric acid, which is subject to hydrolytic ring cleavage to CO_2 and NH_4^+ via hydrolysis of biuret and urea. Our cultures degraded all significant *s*-triazines in real wastewater. But the system was not practicable because the specific activities of some enzymes were too low, because of inhibition by salt in the wastewater and because expensive carbon sources were necessary. Improved planning for enrichment cultures is seen to be necessary and this depends on adequate knowledge of the chemistry of the wastes.

2. INTRODUCTION

The term 'biodegradation' (microbial degradation) is new [1], though a glance in the literature (e.g., [2]) shows that biodegradation is often a synonym for the term 'catabolism', which dates from 1876 [3]. But catabolism (and thus biodegradation) is old, presumably dating back to the growth of the first living cells about 4×10^9 years ago [4–6]. The age of the biosphere and its proven [7–9] and continued ability to evolve in a changing world [6,10,11] allow us to hope that the modern influx of xenobiotic compounds will be met by evolution of new degradative abilities. This guarded optimism (e.g., [12]) is examined here in the development of a biological treatment for the recalcitrant [13; cf. 14] *s*-triazine by-products from

Correspondence to: Alasdair M. Cook, Dept. of Microbiology, Swiss Federal Institute of Technology, CH-8092 Zürich, Switzerland.

the synthesis of the *s*-triazine-herbicides (Fig. 1).

The term 'xenobiotic' (foreign to the biosphere) has a range of meanings and implications revolving around the chemical industry, its products and by-products [24]. Although xenobiotic products need not be a problem [24,25], substances such as DDT can be emotive topics when the control of malaria conflicts with the survival of bird species [e.g., 26]. I wish to approach a problem with a less dramatised group of xenobiotic compounds, the *s*-triazines (Fig. 1).

Whereas a few *as*-triazines (asymmetrical triazines) are known to occur naturally (e.g., the antibiotic fervenulin [27]), *s*-triazines (symmetrical triazines) are generally regarded as xenobiotics [15]. OOOT (cyanuric acid; see Fig. 1 for abbrevi-

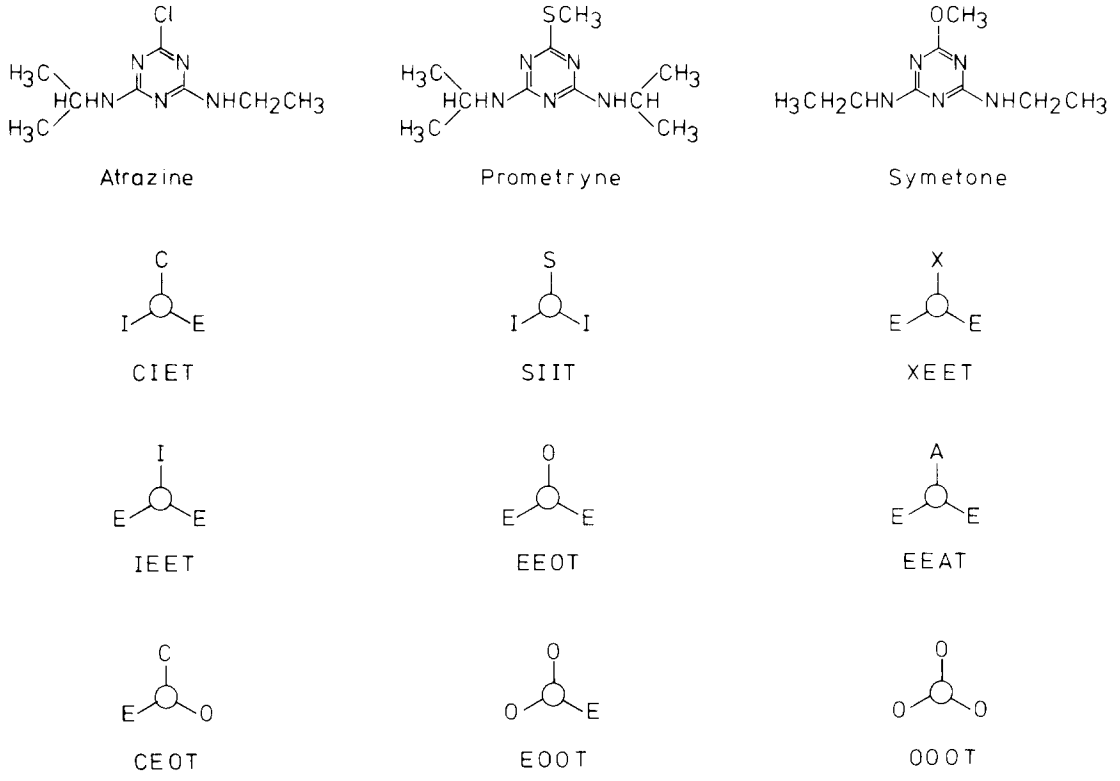

Fig. 1. Representative *s*-triazine herbicides and by-products from their syntheses. The term '*s*-triazine' means symmetrical triazine. Many *s*-triazine herbicides are available commercially, the variety representing in part competing products from different manufacturers, different target plants and the responses of manufacturers to different climates (e.g., [15,16]). Three different types are shown in the first row, and atrazine seems to be the most important *s*-triazine herbicide [16].

The nomenclature of the *s*-triazines is uninformative if using any common names [17–19] and clumsy if using systematic names (atrazine is 6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine). So we developed semi-systematic abbreviations based on the substituents on the ring: A, amino; B, butylamino; C, chloro; E, ethylamino; H, hydro; I, isopropylamino; M, methylamino; O, hydroxy; P, cyclopropylamino; S, methylthio; T, triazine ring structure; X, methoxy. The sequence of the letters for substituents is usually in order of descending molecular mass, except for C, S and X, which always have priority because they so dominate the properties of the compound. The system allows the structure to be deduced from the abbreviation and the abbreviation to be deduced from the structure [20]. It should be noted that the formal use of 'hydroxy' substituents is for convenience in the system and that the keto tautomer predominates in aqueous solution [21].

In Fig. 1, the second row gives a formalised version of the herbicides shown in the first row as well as the semi-systematic abbreviations. Major by-products from syntheses are given in rows 3 and 4: for simplicity, only one homologue is shown though several may be present [13,22, 23]. Information on these and similar compounds and on any putative biodegradation can be obtained from Chemical Abstracts by use of the Substance Index via the Formula Index.

ations) has been claimed [28,29] and refuted [30] as a naturally occurring compound, but Stoks and Schwartz [31] show how easily OOOT can be produced as an artefact during the examination of natural samples (meteorites). So it is feasible that OOOT (and amino analogues) arises naturally from the action of e.g., forest fires and vulcanism on soil. Leaving speculation aside, large quantities of *s*-triazines enter the environment due to their commercial use as, e.g., bleaches and disinfectants (50 000 tonnes world production in 1977) [32], dyestuffs [33], explosives [34], resins and polymers (450 000 tonnes world production in 1976) [35,36], and pesticides, especially herbicides (34 000 tonnes used in the U.S.A. in 1972) [16]. The fate of the *s*-triazine herbicides in the environment is partially defined, and their use has been commercially justified [37]. *s*-Triazines do not accumulate in the environment [37], although Korte [38] does ascribe a relative persistence to them, and their possible entry into groundwater is now being examined [39]. It is this relative persistence that causes a problem, not, apparently, with the large amounts of herbicides, which are spread at low concentrations over wide areas, but rather with the disposal of wastes from the chemical syntheses, i.e., high concentrations at point sources.

The aim of this review is to examine the sequence of steps involved in developing a specific biological treatment for compounds which are classified as recalcitrant. This involves (a) available, routine analytical chemistry to quantify and define the biological reactions; (b) the enrichment of degradative organisms; (c) the analyses of growth physiology of these organisms and of mass balances of metabolism in order to confirm degradation; and (d) examination of the biochemistry of the degradative pathway. The logic of the development is then evaluated in hindsight, after having applied the system to treat real waste: technical aspects of the treatment of wastes are discussed elsewhere [40].

3. CRITICAL ASPECTS OF EXPERIMENTAL TECHNIQUE

3.1. Materials

One must be aware of the real or potential toxicity of xenobiotics, when working with these chemicals, so safety regulations must be adhered to. In contrast to commercial chemical products, wastes from the synthesis of xenobiotics will possibly carry no warning label. Wastes are not quality-controlled, may vary widely in composition from batch to batch, and may contain unknown components (see section *7.2*).

Another problem is the purity of individual chemicals, whether standard reagents or xenobiotics. It is essential to confirm the identity and purity of the xenobiotics under study, regardless of how difficult they are to obtain: we usually received pure chemicals [17,20], but one bottle contained none of the named compound. Mass spectrometry, elemental analysis and a chromatographic analysis are examples of complementary techniques available to confirm identity and purity. Further purification may be necessary prior to biological experiments, especially if the use of indirect methods is contemplated.

3.2. Methods

Indirect tests are common in microbiology. Growth, for example, is often measured as light scattering, and this can be an accurate measure of growth under defined conditions with proper calibration [41,42]. Turbidity in media containing xenobiotics can be due to growth, precipitation or a storage polymer in the cells. We therefore measure protein concentration (section *5.2*), because it is one direct measure of growth [43,44]; we chose protein (instead of e.g., DNA [43,44]) because our research often requires comparisons of enzyme specific activities.

Routine enzyme assays are frequently indirect, e.g., based on the oxidation of NADH. This is accurate because the chemical nature of substrate, product and cofactor [45–47] is defined and the reaction stoichiometry and the molar absorption coefficient of the cofactor are known. This body of knowledge, built on research over 50 years, is usually lacking in work on xenobiotics, so that there is usually no basis for the use of indirect assays in research with xenobiotics, because the identities of substrate and product, cofactor and indeed enzyme, have to be proven before an indirect assay can be validated.

Several surveys ([18,48–50]; see also [38,51]) of papers on the biodegradation of xenobiotics reveal thorough work (e.g., [52,53]; see also [25]) and an over-abundance (60%) of papers whose results are insufficient to support the claims made (see Table 1). This over-abundance stems from a lack of analytical data and quantitative controls. So, if the necessary direct determinations for new work cannot be done with existing methodology, old methods must be adapted (e.g., [69,70]) or new ones developed (e.g., [17,71–73]). Our research would have been practically impossible to validate if we had not had available routine analyses which could be adapted to different samples [13,17,22]. Environmental microbiology depends on analytical chemistry to complement the biology.

Table 1

Data from some papers claiming degradation of *s*-triazines in culture

The only validated claims are those from papers with valid analytical chemistry and controls for growth yields. This table is adapted with permission from Cook and Hütter [18].

Triazine and (days of incubation)	Quality of growth medium [a]	Growth yield [b] (%) per mol of		Assay [c] of			Reference
		N	C	Substrate		Product	
				Purity	Utilisation		
8 compounds [d] (1)	+	100 (+)		+	+	+	[18]
CEET [e] (11)	+		+ [f]	+	+	+	[52]
XEET [e] (7)	–	1 (–)	10 (–)	–	–	–	[52]
CEET [e] (nd [g])	–	nd (–)		–	–	–	[54]
CEET [e] (nd)	–	0 (–)		–	–	–	[55]
CEET [e] (15)	–	nd (–)		–	–	–	[56]
CEET [e] (10)	– [g]	> 400 (–)		–	+	–	[57]
CEET [e] (14)	–	0 (–)		–	+	–	[58]
CIET [d] (18)	–		nd (–)	–	–	–	[59]
CIET [e] (32)	+		+ [f]	+	+	+	[60]
SIIT [d] (21)	–	4 (–)		–	+	+	[61]
SIIT [d] (10)	+	350 (–)		–	+	+	[62]
SIIT [e] (12)	–	nd (–)		–	+	–	[63]
OOOT [e] (9)	+	100 (+)		–	–	–	[64]
OOOT [e] (28)	+	nd (–)		+	+	+	[65]
OOOT [e] (1)	+	100 (+)		+	+	+	[66]
OOOT [d] (7)	–		< 10 (–)	+	+	–	[67]

[a] Growth medium is classified as adequate (+) or inadequate (–). Adequate medium has the *s*-triazine in solution, thus facilitating valid experimentation (sections *3.2* and *4.1*). Furthermore, the molar ratio of total combined carbon to total combined nitrogen in the growth medium is above 12 for aerobic, nitrogen-limited growth and below 8 for carbon-limited growth [25]. Inadequate media do not meet one or both of these criteria and sometimes have additional sources of the so-called limiting nutrient.

[b] The growth yield is expressed as % of standard values (section *5.2*) or of the appropriate control. Fungal protein is calculated from dry weight using the factor 0.5 (section *5.1.2.*). The presence (+) or absence (–) of growth controls is indicated.

[c] The (+) indicates that a valid assay or bioassay is described. These data are only of value in the five cases where a complete mass balance is established: in [57], the organisms were obviously utilising extraneous nitrogen, whereas in [58] there was no growth attributable to the *s*-triazine; in [59] there is obviously growth on impurities, whereas the degradative pathway postulated in [61] consists of standard impurities in the substrate, so application of Occam's razor [68] in the absence of data on substrate purity leads me to disregard these results.

[d] Bacterial metabolism.

[e] Fungal metabolism.

[f] This special case is discussed in section *5.1.4*.

[g] No data given.

4. ENRICHMENT AND ISOLATION OF BIODEGRADATIVE ORGANISMS

The search for the biological catalyst(s) is the first biological step in a project to degrade xenobiotics. It is not an easy initiation into biodegradation, because one of the arts of microbiology is to isolate an unknown organism. Enrichment and isolation of organisms can be facilitated by understanding the basic tenets of microbial physiology (section *5*), community structure [74], evolution [7], the neodarwinian synthesis and the horizontal transfer of genes [8,9,75,76], and analytical chemistry (section *3.2*).

In a review of practical aspects of the enrichment of microorganisms to degrade xenobiotics ([25]; see also [77]) we stress the need for a *clearly defined aim* to facilitate the choice of experiments and allow the results to be evaluated.

The herbicides CEET and CIET and the by-products from their syntheses are not degraded significantly by activated sludge [13,78]. The biological treatment of these by-products requires that some eight compounds or groups of homologues (Fig. 1; some 20 compounds in all) be biodegradable. So *our aim* simplified to a requirement to degrade eight compounds or groups. Each compound should be quantitatively degraded to cell material and inorganic species, the reaction should be fast, and pure cultures were requested.

The following sections in this chapter deal firstly with background information largely from other groups (sections *4.1*, *4.2.1*), and this reveals a biodegradative potential in the biosphere. Our own work (sections *4.2.2–4.3*) is followed by a rationale of approaches to enrichments (section *4.4*).

4.1. Enrichments with a carbon limitation

The *s*-triazines related to herbicides (Fig. 1) are poor sources of carbon because the ring carbon atoms are at the oxidation level of CO_2 [79] and the side-chains are short. Low solubility and a very slow rate of dissolution of particles further limit the amount of carbon available to microorganisms. However, enrichments with carbon limitation are a standard approach to biodegradation (e.g. [80]) and several groups have tested the hypothesis that an *s*-triazine can be utilised as sole carbon and energy source for growth.

Kaufman et al. [81] set up selective minimal medium with limiting CEET and a soil inoculum, and isolated a fungus (*Aspergillus fumigatus*, now lost; J.S. Karns and P.C. Kearney, personal communication, 1986), which utilised the side-chain of CEET for growth (section *5.2*). Giardina's group [82–84] is studying an aerobic bacterium, *Nocardia* sp., which was isolated from CIET-treated soil; there was no successful enrichment from untreated soil for the degradation of CIET. The strain may utilise one or more side-chains of CIET aerobically for growth, but the physiological data are inconclusive (sections *5.1.2* and *5.1.4*). Behki and Khan [85] have now isolated *Pseudomonas* spp. which can utilise CIET as a sole source of carbon and energy for growth. Only soil pretreated with CIET yielded enrichments to degrade CIET, but here too, the physiological results are inconclusive (section *5.1.4*). In contrast to utilisation of the side-chain, Jessee et al. [67] have isolated an unidentified anaerobic bacterium, which is claimed to utilise the ring carbon of OOOT as a major source of carbon and energy under mixed substrate conditions: however, the mass balance for carbon (7% recovery; see [79]) is unsatisfactory, so more evidence is required to establish the claim.

The anaerobic degradation of the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by sewage has been described [86], but the products formed were excreted and not utilised for growth. Another saturated triazine (hexahydro-1,3,5-triethyl-1,3,5-triazine) may be degraded by *Pseudomonas* spp. [87], but no attempt has been made to distinguish between spontaneous and biological degradation.

These papers [81,82,85] generally give an impression of experimental difficulties with slow (often weeks) and incomplete conversions to other *s*-triazines; our own experiments yielded no enrichments to utilise these compounds as carbon sources. The use of the word 'recalcitrant' [14] for *s*-triazines can be understood. But a variety of these compounds can now be at least partially degraded, so *s*-triazine-degrading enzymes must exist in fungi and bacteria.

4.2. *Utilisation of* s-*triazine nitrogen*

4.2.1. *Utilisation without enrichment*

In contrast to the small amount of carbon in *s*-triazines, there is a high level of nitrogen available to support growth of organisms which have suitable degradative enzymes (see Fig. 1). Three fungi (two *Penicillium* spp. and a *Hormodendrum* sp.; Table 2), isolated to utilise biuret ($H_2NC(O)NHC(O)NH_2$) as a nitrogen source, also utilised OOOT as a nitrogen source [64] (see Table 1). A fungus (*Stachybotrys chartarum*), obtained non-selectively (from a culture collection), degraded OOOT in complex medium [65] and Zeyer et al. [66] obtained non-selectively a fungus (*Sporothrix schenckii*) that quantitatively degrades

Table 2

Organisms degrading *s*-triazines in pure culture and in defined medium

Organism	Moiety utilised	Triazine in enrichment	Substrate spectrum [a]	Reference
Utilisation of carbon				
Aspergillus fumigatus	Side chain	CEET	CEET	[52,53] [81]
Nocardia sp.	Side chain	CIET	CIET [b]	[82,83]
Pseudomonas sp.	Side chain	CIET	CIET [b], CIAT [b], CEAT [b]	[85]
Unidentified	?	OOOT	OOOT	[67]
Fungal utilization of nitrogen				
Penicillium spp.	Ring	None	OOOT	[64]
Hormodendrum sp.	Ring	None	OOOT	[64]
Sporothrix schenkii	Ring	None	OOOT	[66]
Bacterial utilisation of nitrogen				
Klebsiella pneumoniae strain 99	Ring and amino group	OOOT	OOAT, OOOT	[18]
Klebsiella pneumoniae strain 90	Ring and amino group	OOAT	OOAT, OOOT	[18]
Pseudomonas strain A	Ring and amino groups	OAAT	AAAT, OAAT, OOAT OOOT, CAAT, PAAT, POAT [c]	[18,90]
Pseudomonas strain D	Ring and amino alkyl group	IOOT	IOOT, OAAT, OOAT OOOT	[18]
Pseudomonas strain F	Ring and amino alkyl group	IOOT	IOOT, OAAT, OOAT OOOT	[18]
Rhodococcus corallinus strain 11	Ring and amino groups	CEAT	CEAT, CAAT, PAAT POAT [c], AAAT, OAAT, OOAT OOOT	[91]
Pseudomonas sp. strain C38E	Ring, amino and amino alkyl groups	EEAT	EEAT, IEAT, IIAT EOAT, EOOT, OOOT	[92]
Unidentified strain C3E	Ring	COOT	COOT, OOOT	[92]
Utilisation of sulphur				
Pseudomonas sp. strain 26	Methylthio side chain	SIIT	SIIT, SIET, SIOT	[50]

[a] Utilisation or conversion is complete, unless otherwise indicated.
[b] Incomplete conversion of substrate to product(s).
[c] Analogues are also degraded; at least M, E and I in place of P.

OOOT as the sole source of nitrogen.

The frequency with which OOOT-degradation is observed in cultures obtained under non-selective conditions suggests a widespread natural occurrence of OOOT-catabolism in fungi. This implies a widespread occurrence of OOOT in the environment, but it is still uncertain whether OOOT is a natural or a commercial product (section *2*).

Zeyer et al. [88] observed that many microorganisms from non-selective enrichments can ring-deaminate low concentrations (μM) of OAAT in complex medium.

Most organisms mentioned in this section (*4.2.1*) grow slowly (incubations of 1–4 weeks [66]) and have narrow or unknown substrate ranges; the few data available were difficult to collect, due to the very cumbersome analytical methods then available (e.g., TLC-autoradiography). Nevertheless, in contrast to the carbon-limited enrichments, enzymes for ring cleavage and removal of side-chains would appear to be widespread, because organisms containing them were obtained without specific selection.

4.2.2. Enrichments to utilise s*-triazines from wastewaters*

4.2.2.1. Simple batch cultures

With this background (sections *4.1* and *4.2.1*), the industrial requirement for rapid degradation of a wide range of *s*-triazines led us to strict nitrogen-limited enrichment cultures containing inocula from soils long exposed to large amounts of *s*-triazines and from which several bacteria were isolated (Table 2). These organisms grow rapidly in *s*-triazine-minimal-salts medium containing a carbon source and quantitatively utilise the *s*-triazine nitrogen source: they fall into three taxonomic groups.

(a) Bacteria attributed to the genus *Klebsiella* (Table 2) were isolated from municipal sewage. These strains degrade only two substrates (OOOT and OOAT; Fig. 5), but they utilise them aerobically and anaerobically [18,89] and they grow one or more orders of magnitude faster (e.g., for strain 99 in OOOT-glucose-mineral medium, $\mu = 0.6$ h^{-1}) than earlier (fungal) isolates.

(b) A second group consists of three bacteria attributed to the genus *Pseudomonas*, which may belong to the *acidovorans* group because, in addition to published data [18], they contain ubiquinone Q8 (G. Auling, personal communication, 1986). *Pseudomonas* sp. strain A (NRRLB-12227) was isolated from municipal sewage. The characteristic of this strain (beside ring cleavage) is the wide range of quantitative ring deaminations catalysed (e.g., conversion of PAAT to POOT) but the failure to remove other substituents from the ring (e.g., the alkylamino group from POOT) (Table 2). Strain A has a wide substrate range and grows moderately rapidly ($\mu = 0.4$ h^{-1} in OAAT-lactate-minimal medium).

The *Pseudomonas* strains D (NRRLB-12228) and F (NRRLB-12229) are similar to one another and grow moderately rapidly ($\mu = 0.3$ h^{-1} in IOOT-lactate-minimal medium), but strain F produces much more slime than strain D. Strains D and F were obtained only from soil exposed to *s*-triazine herbicides for at least 3–7 years. These bacteria have a limited substrate range, but this includes (besides ring cleavage) a very important reaction, the removal of the alkylamino side-chain (e.g., from POOT). This activity complements the reactions of strain A, enabling the total degradation of multiply substituted *s*-triazines, e.g., PAAT [20]. This coupled metabolism occurs only in non-growing cells; strains A and D (or F) do not grow well together, for reasons that are not understood.

(c) The organism with the widest known substrate spectrum for *s*-triazines is *Rhodococcus corallinus* strain 11 (NRRLB-15444R). This strain was obtained by batch enrichment only from soils exposed for up to 20 years to high levels of *s*-triazine herbicides. This organism can dechlorinate a chloro-*s*-triazine as well as deaminating many compounds and catalyzing ring cleavage; the alkyl amino side-chain, however, is not removed. In contrast with *Pseudomonas* strain A, this Gram-positive organism grows well in co-culture with strain D and gives total conversion of CEAT to cell material and CO_2. Strain 11 grows slowly ($\mu = 0.04^{-1}$ in CEAT-glycerol-minimal medium), though the specific activity of the dechlorination is high (section *5.2*).

The organisms in these three groups (*a–c*) fail to grow on or transform most of the *s*-triazines in wastewater [13]. Another series of nitrogen-limited batch enrichments was done with a wider range of inocula: (a) more soils with long-term exposure to *s*-triazines; (b) the same soils packed in percolation columns and treated for several months with *s*-triazine-containing medium (e.g., EEOT-minimal medium with carbon sources); (c) activated sludge from a plant receiving *s*-triazine-wastes; and (d) neglected corners in the apparatus used to synthesise the *s*-triazine herbicides. New organisms were isolated with metabolic patterns which we had already observed, but no new degradative abilities were obtained [92]. So we concluded that the practical limits of simple batch enrichments had been reached.

4.2.2.2. Long-term selection procedures

We set up two non-sterile experiments to try to develop the required degradative abilities by long-term selection. It was hoped that a heterogeneous inoculum, which contained the genetic potential for the synthesis of large numbers of *s*-triazine-degrading enzymes representing all the necessary reaction types theoretically required to metabolise the undegraded compounds, would readily lead to the development of degradative organisms, presumably via mutation and gene transfer among organisms [92]. A continuous culture and an extended series of batch cultures were used, and both experiments gave similar results. Only the latter experiment is described here.

Wastewater was the source of nitrogen and several sources of carbon were used. The inoculum contained six cultures form this laboratory (strains A, D, F, 11, 90 and 99; see Table 2) and material from all available sources exposed to *s*-triazines (section *4.2.2.1*). At the start of the experiment, only EOOT, a substrate for strains D and F, was quantitatively degraded (Table 3). Organisms were, however, utilising one or more *s*-triazines for growth and thus presumably producing a steady supply of mutants (e.g., [25]) under nitrogen limitation conditions designed to select for utilisation of further *s*-triazines as nitrogen sources.

An event between days 15 and 17 led to the quantitative disappearance of EEAT (Table 3), and an organism (*Pseudomonas* sp. strain C38E; Table 2) was isolated which deaminates EEAT to EEOT [92]. The development of an activity to degrade CEOT was slower (Table 3). No stable pure culture was found to utilise CEOT for growth, although mixtures of pure cultures do utilise the compound [92]. The development of degradative enzymes for EEOT or IEET was even more gradual (Table 3): in these cases, neither pure cultures nor satisfactory defined mixed cultures of degradative organisms were obtained [92].

We had attained our goal of a (mixed) culture able to degrade all the significant components in wastewater. In addition, an unidentified bacterial pure culture able to degrade COOT was obtained (Gram-negative, non-motile rod, strain C3E; Ta-

Table 3

Development of degradative activities for representative *s*-triazines in batch cultures subject to weekly subculture into medium with wastewater as the limiting nitrogen source

s-Triazine	Substrate disappearance (%)				
	5 days	17 days	54 days	88 days	177 days [a]
EOOT	100	100	100	100	100
CEOT	0	0	0	95	100
EEOT [b]	30	30	30	50	80
EEAT	0	100	100	100	100
IEET	0	0	0	50	$\leqslant 100$

[a] Age of culture.

[b] EEOT tends to give oversaturated solutions, so small reductions in concentration can be artefacts: a further complication is that EEOT is a product from the degradation of more complex *s*-triazines (e.g., EEAT [92]), so low reductions in concentration may mask a flux of EEOT.

ble 2). Degradation of CIET (atrazine, an insignificant component of the waste) as a source of nitrogen was also observed, but this culture was lost [92].

4.3. Enrichments with a sulphur limitation

A limited number of *s*-triazine herbicides and by-products from their production contain sulphur [15,22] (Fig. 1). None of these compounds is known to be a carbon or nitrogen source for growth [18,50] (Table 1), so enrichments were prepared to test the hypothesis [93] that SIIT and SIET are sulphur sources for growth [50].

Work with a sulphur limitation requires care to eliminate spurious sources of sulphur; we work with purified sulphur sources at an initial concentration of 20–30 μM [49,50,94,95], with re-rinsed glassware and with incubators free of volatile sulphur sources. We obtained several unidentified bacteria which were able to utilise SIET as sole source of sulphur. One organism (*Pseudomonas* sp. strain 26) could utilise SIIT, SIET and SIOT (Table 2).

These organisms were obtained only from inocula with an extensive history of exposure to *s*-triazines [50]. The claim [93] that a culture-collection strain of *Aspergillus niger* [96] utilizes SIIT as a sulphur source appears to be due to utilisation of traces of contaminant sulfur [50] (section *5.1.3*).

4.4. Rationale

Enrichment cultures are about a century old [97], but it seems to be frequently overlooked that selective enrichment cultures yield better strains (or mixtures of strains) for specific purposes than do non-selective enrichments. An organism selected for its ability to grow on a nutrient agar plate represents about 3000 genes [98], of which perhaps 10% will code for degradative enzymes (i.e., 75 substrates each with a 4-gene pathway) [99]: the chance that a xenobiotic will be degraded by these 300 proteins is low. In contrast, 1 g of fertile soil represents about 10^8 organisms [97] or a potential of some 10^{10} degradative enzymes: this inoculum is statistically more likely to degrade the xenobiotic. Sewage sludge, with some 100-fold higher populations than soil [100] and selected to degrade wastes, offers a still better chance to degrade the xenobiotic under study. These chances can be further improved by chosing inocula pre-exposed to the xenobiotic, such that evolution of functional degradative enzymes may occur prior to the experiment: this enrichment requires only a batch culture! When the batch enrichment fails, the more complex methodology of continuous culture can be used to obtain biodegradation [25,92,101].

Another point is which nutrient to limit in which minimal medium? As seen above, a standard answer, carbon, is not always suitable, and nitrogen or sulphur limitation can be successful (see also [25]).

I have avoided co-metabolism [25,102]. The concept has been criticised, because it is not one idea, but many [103]. One of those ideas has been defended [104,105], namely 'transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound'. This idea places co-metabolism at the level of the individual organism, effectively derived from the broad specificity of some enzymes. In enrichment cultures of the types described above, co-metabolism is indistinguishable from metabolism and assumes importance when pure cultures are required.

5. GROWTH PHYSIOLOGY

Growth is a complex topic (e.g., [106,107]), but the information in Table 1 demonstrates that some basic concepts in growth physiology and their relevance to the degradation of xenobiotics need to be discussed. In particular, the growth medium is often seen to be inadequate [48,49] (Table 1). So I believe that the molar growth yield of an organism must be explored first, to check the adequacy of the medium and the organism. Only then can the growth rate be examined meaningfully.

5.1. Molar growth yields

5.1.1. Carbon sources

Cell growth is usually directly proportional to

the amount of a limiting nutrient until some other component becomes limiting [108], but this proportionality must always be established, and not just assumed. This is shown in an examination of the carbon-limited growth of an unidentified bacterium, strain PA14 (which degrades sulphanilate, 4-aminophenyl sulphonate), and of a spontaneous mutant, S4 (which grows to a higher density with the same amount of sulphanilate). Both organisms had the same yield with succinate as carbon source, and growth was proportional to the concentration of substrate supplied. However, whereas the mutant grew with a yield of 35 g of protein/mol of sulphanilate (Fig. 2), strain PA14 degraded the substrate but did not grow proportionally to the carbon atoms supposedly consumed (T. Thurnheer, unpublished results) [109]. The results from strain S4 (and of strain PA14 utilising succinate) indicate molar growth yields of about 6

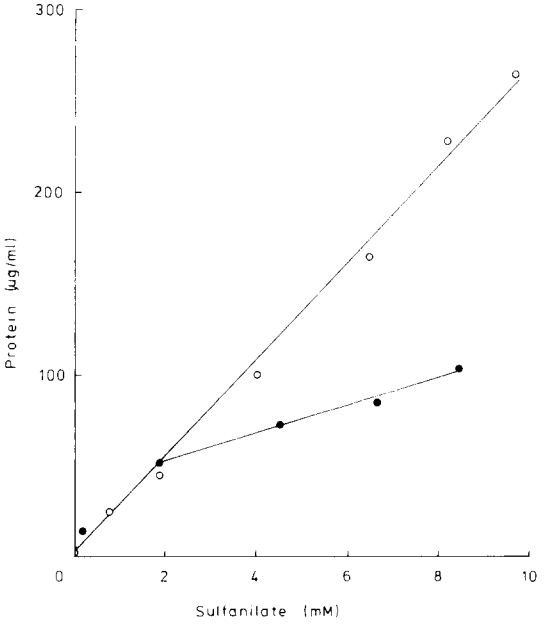

Fig. 2. Growth of wild-type bacterium strain PA14 and mutant strain S4 with sulphanilate as sole source of carbon and energy in mineral medium. Growth (as protein) was measured after disappearance of substrate: no further growth of strain PA14 in sulphanilate-mineral medium was observed when cultures were left for longer periods or supplied with more vitamins or with more trace elements (T. Thurnheer, unpublished data). ○, strain S4; ●, strain PA14.

g of protein/mol of carbon (a commonly observed value [25]) and this value can be calculated to be unexceptionable:

Cell dry matter = approx. 50% (w/w) protein [110]
Cell dry matter = approx. 50% (w/w) carbon [110]
Respiration during growth = approx. 50% of the carbon source [108]
Growth yield of strain S4 = 6 g of protein/mol carbon
or = 12 g of dry matter/mol carbon
or = 6 g of carbon recovered/mol carbon
Further, respiration = 6 g of carbon released/mol carbon
Mass balance = 12 g of carbon/mol (i.e., 100%)

Thus, a simple experiment (Fig. 2) yields significant information. Firstly, strain S4 is seen to grow as a linear function of the substrate supplied, so growth experiments with, e.g., 3 mM-sulphanilate, can be expected to give logically interpretable data (in contrast to data from the same experiment with strain PA14). Secondly, the slope of the line implies that all carbon is being utilised for energy generation and growth, and that a provisional mass balance involving complete conversion of substrate to natural products can be used as a working hypothesis.

5.1.2. Nitrogen sources

Our work with *s*-triazines was usually nitrogen-limited, because most successful enrichment cultures were nitrogen-limited (section *4.2.2*). All strains tested have linear responses of growth to nitrogen supply and an average yield value is 50 g of protein/mol of nitrogen [18]. This value is commonly observed [25], and is unexceptionable for nitrogen-assimilatory growth:

Cell dry matter = approx. 14% (w/w) nitrogen [110]
Cell dry matter = approx. 50% (w/w) protein
Growth yield = 50 g of protein/mol nitrogen
or = 100 g of dry matter/mol nitrogen
Mass balance for nitrogen = 14 g of nitrogen/mol (i.e., 100%)

Our results show that different bacteria can have different characteristic molar growth yields. Whereas strains A, C3E and C38E (Table 2) pro-

duce some 50 g of protein/mol of nitrogen, strains D, F and 11 produce 40 g and strains 90 and 99 produce 65 g [18,91,92]. Presumably, different strains produce different ratios of nitrogen-containing compounds (e.g., protein and cell wall), which lead to different colour intensities with the Lowry-type test we use. Thus, although we have a rule of thumb for growth yields, each strain must be tested individually and compared with appropriate controls.

We find molar growth yields valuable in assessing both our own strains and published data. Growth yields were the first indication we had that strain A (Table 2) utilises all six nitrogen atoms in AAAT but only two from PAAT (Table 4, the two left-hand columns), thus leading to the identification of POOT as a product accumulating from the metabolism of PAAT. Similarly, strain 11 utilises all six nitrogen atoms in AAAT, but only one from CEAT [91], so the growth-yield experiments together with analysis of the products allow preliminary sketching of degradative pathways (Fig. 5 and section *6.2*).

Giardina et al. describe CIET as a nitrogen source for a *Nocardia* sp. in glucose mineral medium in batch culture [83] (section *5.1.4*). 40% of the CIET (initially 0.14 mM) and 0.01 mM CAHT were present when the protein concentration was maximum; other *s*-triazine products are ignored in this calculation, in which $\geqslant 120$ g of protein/mol nitrogen seem to be formed. Whatever source(s) of error causes this overly high yield, it is not the identification of the product, CAHT, which is conclusive. No control experiment with, e.g., ammonium ion as nitrogen source, is available.

5.1.3. Sulphur sources

Another limiting substrate used in work with *s*-triazines is sulphur [50]. The bacteria we isolated have growth yields in the range of 3–6 kg of protein/mol sulphur, which we find to be common [25,94,95]. The comparisons of these yield data with accepted values for the sulphur content of cells is less straightforward than those with carbon and nitrogen limitations, where data for carbon or nitrogen limited cells were used, because the sulphur content comes from cells growing with excess sulphur [110]. It is clear from the few data available [111] that cells alter their distribution of sulphur on sulphur starvation (e.g., small molecules virtually disappear and some proteins are no longer synthesised). For simplicity, economy of a factor 2 in the allocation of sulphur by the cell and a low sulphur growth yield is assumed, to argue that the data are unexceptionable:

Cell dry matter = 1.1% (w/w) sulphur [110]
and in sulphur-limited cells = 0.5% (w/w) sulphur
Cell dry matter = 50% (w/w) protein
Growth yield = 3 kg of protein/mol sulphur
i.e., = 6 kg of dry matter/mol of sulphur
Mass balance for sulphur = 32 g of sulphur/mol (i.e., 100%)

5.1.4. Alternative assay methods

Clearly, growth yield experiments (with appropriate controls) give good preliminary data on the degradation of a compound. However, it is not always feasible to do growth yield experiments of the type shown in Fig. 2. One example of this is found in the experiments with carbon-limited CEET-minimal medium [52]. The sparing solubility of the carbon source (25 μM) means that utilisation of the ethyl side-chain(s) would not support growth visible to the eye. Kaufman et al. [52] followed the fate of ^{14}C-labelled ethyl groups. Radioactivity was recovered largely as CO_2, but the portion in cell material was distributed evenly in protein, lipid and nucleic acid fractions. The alkyl group was thus largely oxidised to provide energy to utilise a portion of the carbon for growth (Table 1). This method of tracing the fate of the alkyl side-chain could be used to advantage in work with a *Nocardia* sp. [83], which appears to degrade CIET with a yield of 29 g of protein/mol of carbon, and in work with *Pseudomonas* spp. [85], where an apparent yield of about 40 g of protein/mol of carbon in strain 194 can be calculated. These growth media obviously contain additional sources of carbon considerably in excess of the amounts of *s*-triazine carbon available, whose fate is thus unknown.

An alternative method to quantify growth is to follow cell numbers, because cell size and contents

Table 4

Extent and rate of metabolism of *s*-triazines and derivatives as nitrogen sources for three strains of bacteria

Cells were grown in mineral medium containing the nitrogen source shown ('substrate') and a carbon source (lactate for strains A and D, and glucose for strain 99). If cultures grew, there was quantitative disappearance of the nitrogen source, though in some cases (marked +) no data are presented because the substrate was impure (POAT, POOT) or for other reasons (see section *5.3*). The molar growth yield ('growth') is expressed here as mol of nitrogen incorporated/mol of substrate. The extent of metabolism is given as mol of NH_4^+ released/mol of substrate by cell suspensions. The specific growth rate (μ) is shown, and those data used with the growth yield (expressed as g of protein/mol of substrate), to calculate the specific degradation rate (mkat/kg of protein) in growing cells [18,20,79].

Substrate	Mol N/mol substrate	Bacterial strain											
		A				D				99			
		Growth (mol N in cells/mol substrate)	NH_4^+ released by non-growing cells (mol N/mol substrate)	μ (h^{-1})	Degradation rate (mkat/kg protein)	Growth (mol N in cells/mol substrate)	NH_4^+ released by non-growing cells (mol N/mol substrate)	μ (h^{-1})	Degradation rate (mkat/kg protein)	Growth (mol N in cells/mol substrate)	NH_4^+ released by non-growing cells (mol N/mol substrate)	μ (h^{-1})	Degradation rate, (mkat/kg protein)
AAAT	6	6	6	0.31	0.2	0	0			0	0		
OAAT	5	5	5	0.42	0.4	5	5	0.29	0.4	0	0		
OOAT	4	4	4	0.42	0.6	4	4	0.29	0.4	4	4	0.31	0.3
OOOT	3	3	+	0.28	0.5	3	3	0.31	0.7	3	+	0.63	0.9
Biuret	3	0	0			3	3	0.26	0.6	0	0		
Urea	2	2	2	0.53	1.4	2	2	0.30	1.0	2	2	0.39	0.9
NH_4^+	1	1		0.52	2.6	1		0.29	1.9	1		0.77	3.2
PAAT	6	2	2	0.15	0.3	0	0			0	0		
POAT	5	+	+			0	0						
IOAT	5	1	1	0.12	0.5	0	0			0	0		
POOT	4	0	0			+	+						
EOOT	4	0	0			4	4	0.28	0.4	0	0		

vary only within a narrow range [107]. However, if viable counts are used, the plating efficiency must be given (or a control experiment provided). Otherwise, the sceptical reader will interpret his calculations from cell numbers to mean, e.g., 2% incorporation of carbon from the xenobiotic into cell material, as we did [25], rather than the 100% incorporation claimed.

5.2. Growth kinetics

Kinetic analysis of quantitative substrate utilization during growth allows a more complete picture of the interaction of the organism with its substrate to be obtained.

We normally observe exponential growth in cultures utilizing *s*-triazines as nitrogen sources (e.g. [18,91]) and some specific growth rates are given in Table 4. The growth rate is not the only important value: the specific degradation rate indicates at what rate a substrate is utilised. Thus one important compound, IOOT (Fig. 1), is degraded at 0.4 mkat/kg of protein by strain D growing with a μ of 0.28 h^{-1} (Table 4). The closely related compound IOAT supports much slower growth (of strain A, 0.12 h^{-1}; Table 4) but the rate of utilisation of IOAT (0.5 mkat/kg of protein) is similar to that of IOOT. A similar calculation shows that the slow-growing strain 11 ($\mu = 0.04$ h^{-1} in CEAT-glycerol-salts medium) has a specific degradation rate for CEAT of 0.3 mkat/kg of protein [91], similar to the degradation rates of the strains mentioned in Table 4.

One tends to anticipate that exponential growth means exponential utilisation of the substrate, and this is often the case, as is shown in Fig. 3. Strain A grows exponentially with ammonium ion, OAAT or AAAT as nitrogen source, but only with ammonium ion or OAAT is growth concomitant with substrate utilisation, as seen in a plot of residual substrate concentration vs. protein concentration (Fig. 3B). The apparently similar growth with AAAT masks a rate of substrate disappearance which is much faster than that required for growth and which leads to the excretion of an intermediate. The intermediate is OAAT which is subsequently utilised.

We have seen the excretion of an intermediate in only four cases (once each with strain A (Fig. 3), strain 11 [91] and strains 90 and 99 [18,79]) but biphasic growth was observed only with strain 99 utilising OOOT, where biuret was excreted and later utilised (Fig. 5). These latter data suggest that strain 99 can synthesise a biuret transport system only under certain conditions, because the

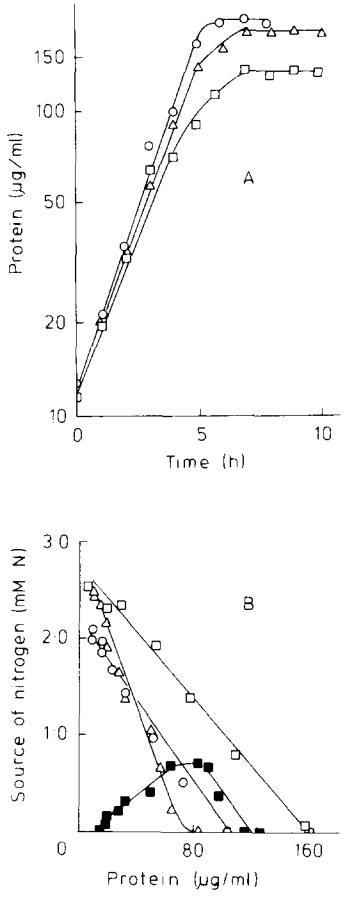

Fig. 3. Growth kinetics of *Pseudomonas* sp. strain A with NH_4^+, OAAT or AAAT as source of nitrogen. Strain A (Table 2) was grown at 30 °C in aerated lactate-mineral medium containing a nitrogen source. Cells were harvested in the late log phase, resuspended in mineral medium and used immediately as inocula for growth experiments in which samples were taken at intervals for determinations of protein, *s*-triazines and NH_4^+ [18,89]. (A) Growth curves with three different sources of nitrogen; the inoculum was grown in homologous medium. (B) Results from the same experiment, but the substrate concentration is plotted vs. the protein concentration. Symbols: ○, NH_4^+; □, OAAT as sole added source of nitrogen; ■, OAAT excreted during growth with AAAT; △, AAAT. The data in (B) are adapted from Jutzi et al. [89] with permission.

organism cannot grow with biuret as a sole source of nitrogen or degrade it in suspensions of non-growing cells (Table 4).

The method of plotting data from a growth experiment (Fig. 3B) readily gives information on the possible existence of an accumulated intermediate. The plot is the kinetic equivalent of the end-point yield data (Fig. 2) and allows the mass balance to be checked throughout growth.

5.3. Suspensions of non-growing cells

The working hypotheses developed during growth yield and growth rate experiments can be tested and extended with work using non-growing cells suspended in buffer. Under these conditions we could demonstrate that *s*-triazine nitrogen is converted to ammonium ion (Table 4). Most of these reactions are quantitative conversions with complete substrate disappearance. Each reaction takes place as readily anaerobically as aerobically, which we interpret to mean catalysis by hydrolases.

Cells suspended in buffer are sometimes sufficiently dissimilar to growing cells that otherwise-undetected intermediates can be observed. Thus strain A (and 99; Table 4), which releases stoichiometric amounts of ammonium ion from OOAT, forms little ammonium ion from OOOT and excretes biuret (section *5.2*). We hypothesise that these cells of strains A and 99 contain no transport system for biuret, which leaks out of the cells on its production by the highly active OOOT-cleavage enzyme (2.9 mkat/kg of protein in extracts of strain A; Table 5). OOAT-deamination is a slower reaction (1.1 mkat/kg of protein in extracts of strain A; Table 5) so there is presumably no accumulation of biuret in the cell in this case.

The information in this section (section *5*) illustrates the tight coupling of the microbiology of degradation with the specific determination of substrates and the identification and quantification of products. Both components are necessary and complementary parts of the project (see section *3.2*).

Table 5

Specific activities in cell extracts and course regulation of synthesis of some *s*-triazine-degrading hydrolases [20,79,89,91,128]

Enzyme substrate	Strain	Specific activity (mkat/kg of protein)	Enzyme synthesis
AAAT	A	2.3	Constitutive
PAAT	A	0.2	Constitutive
OAAT	A	0.4	Constitutive
POAT	A	0.7	Constitutive
OOAT	A	1.1	Constitutive
OOOT	A	2.9	Constitutive
OOOT	99	4.5	No data
IOOT	D	1.4	Constitutive
CEAT	11	0.2	Constitutive [a]
CEAT	11	0.2	Inducible [b]

[a] This enzyme deaminates AAAT and CAAT, as well as dechlorinating CEAT and CIAT.

[b] This enzyme appears to catalyse only the dechlorination of CEAT and CIAT.

6. DEGRADATIVE PATHWAYS OF *s*-TRIAZINES

Biotransformations and metabolism of *s*-triazines in plants, animals, soil, water and microorganisms have been reviewed [15,37,112–119] (see also [120,121]). Reviews of the microbial degradation of the *s*-triazines rely mainly on papers [52,53,60] describing one (or two) reactions (Fig. 4A), because these results are obviously sound (Table 1). Our data allow the assembly of complete degradative pathways for several *s*-triazine pesticides and their metabolic and synthetic by-products, often with complete mass balances for individual reactions.

6.1. Oxidative dealkylation of side-chains

The first reliable work on the catabolism of the *s*-triazines [52,53,60] (cf. [91,122]) was complicated by the low solubility of the substrates (25 μM for CEET and 120 μM for CIET) and the difficulties of isolating sufficient product for physical identification. Nevertheless, CEAT was conclusively identified as one of two products from CEET degradation (Fig. 4A), presumably formed by oxidative *N*-dealkylation [115]. The ethyl group(s) was

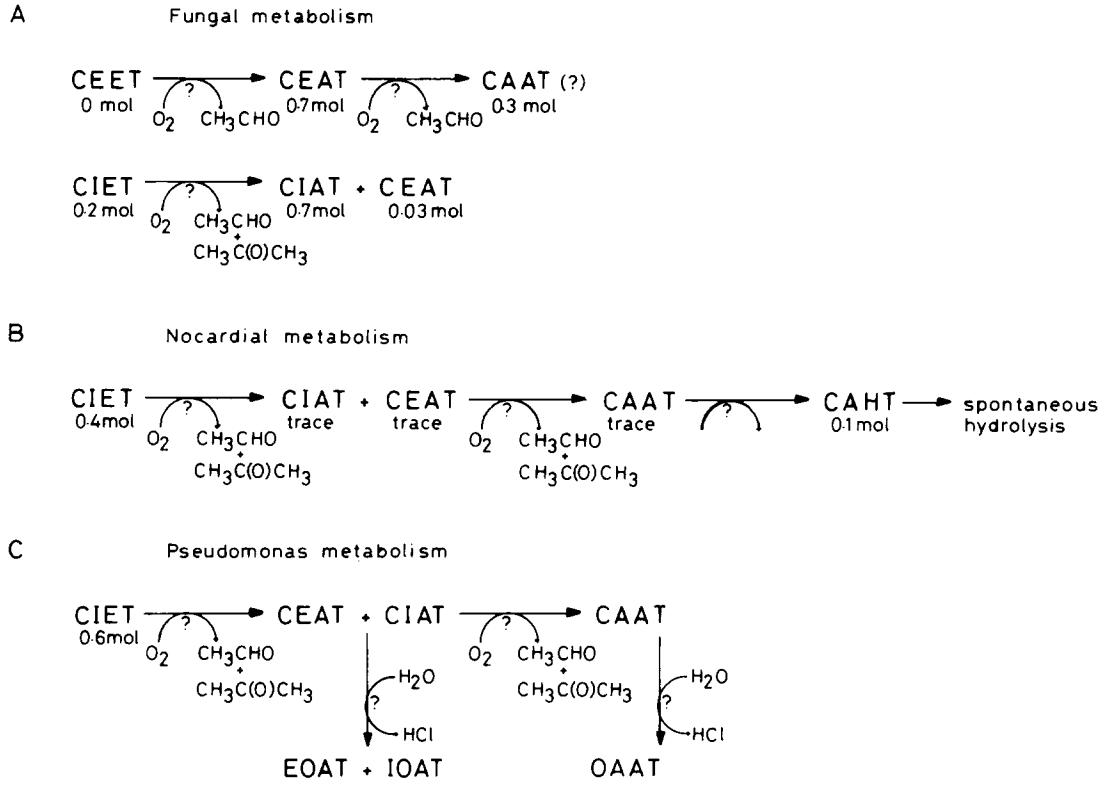

Fig. 4. Degradation of CEET and CIET by the fungus *Aspergillus fumigatus*, and of CIET by a *Nocardia* sp. and by *Pseudomonas* spp. These reactions have been studied in whole cells only and assumptions or gaps in our knowledge are indicated by question marks. The stoichiometry of each reaction is indicated in the figure as the products from 1 mol of substrate. (A) Fungal metabolism. The identity of CEAT formed from CEET has been established. The postulate that CAAT is formed by a second oxidative dealkylation is weak (section *6.1*). (B) Nocardial metabolism. The biological reactions shown depict only part of the metabolism suspected by Giardina's group [83,84] (section *6.3*). They also report spontaneous degradation of CAHT, CAAT, CEAT and CIAT; whereas CAHT is predictably unstable [21], we find CIAT, CEAT and CAAT to be stable in sterile growth medium [90,91]. (C) *Pseudomonas* metabolism. The metabolites have been conclusively identified, but there are no unified data on their distribution after the incubations, which last up to 5 weeks [85].

metabolised further (section *5.1.4*). CIET was also dealkylated by this system (Fig. 4A) [60]. The identification of the second product from CEET as CAAT has been claimed but not confirmed [123] (cf. [114]), and no further data can be collected because the strain has been lost (section *4.1*). Putative oxidative dealkylations occur in bacteria also and the pathways involve CAAT as an intermediate (Fig. 4B, C).

6.2. Hydrolytic reactions

The *s*-triazine products from oxidative dealkylation are all now biodegradable to CO_2 and ammonium ion (Fig. 5). However, it would seem more sensible to discuss the degradation of simpler *s*-triazines first, as an introduction to the degradation of the more complex compounds, which are funnelled to one central metabolite prior to ring cleavage.

AAAT is degraded by *Pseudomonas* sp. strain A (Table 2) in a six-step sequence to CO_2 and NH_3 (Fig. 5). Each step in the sequence has been examined individually in partially purified protein fractions in order to identify conclusively the organic and inorganic products and to confirm stoicheiometry. There is no requirement for molecular oxygen in these reactions [79,89]. The

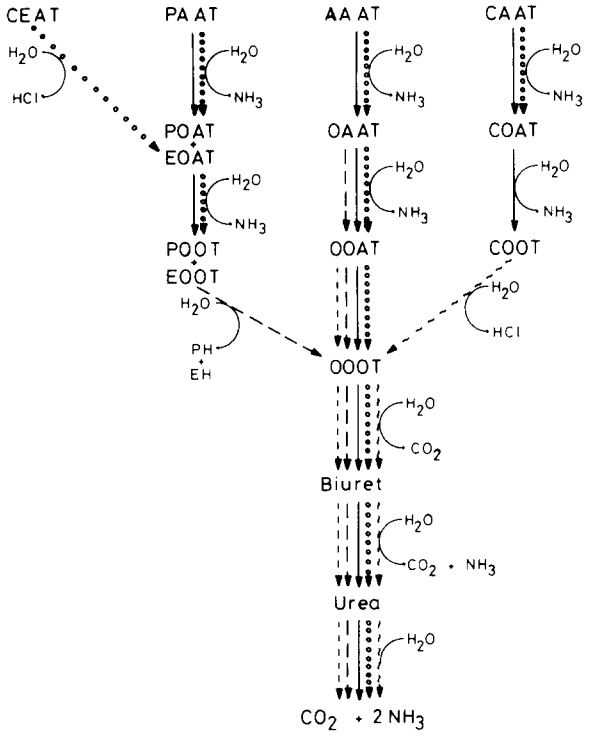

Fig. 5. Pathways of bacterial degradation of derivatives of *s*-triazine herbicides and larvicides. Each reaction shown is known to occur and, except COAT to COOT and COOT to OOOT, has been studied in detail in separated protein fractions where 1 mol of substrate was converted quantitatively to one mol each of products which were identified conclusively. Under physiological conditions (we used pH 7.2) NH_3 is present as NH_4^+, but the figure can be more simply drafted with balanced reactions using the NH_3 moiety. No organism contains all the reactions shown, and complete degradation of many compounds requires communities. The arrows indicating reactions also represent the bacterial strain (Table 2) containing the enzyme: (———), A; (— — —), D; (· · · · · ·), 11; (– – – – – –) 99; (~ ~ ~ ~ ~ ~ ~) C3E.

pathway, or sometimes only the lower parts of it, is found in other Gram-negative bacteria (*Pseudomonas* and *Klebsiella* spp. strains D, F, 90 and 99; Table 2), a Gram-positive bacterium (*Rhodococcus corallinus*; Table 2) and in a fungus (*Sporothrix schenckii*; Table 2) [66,79,88,89,91,124]. Thus, the sequence must be regarded as an established degradative pathway.

Two basic reaction types are involved in the degradation of AAAT (Fig. 5) [79,89]. The first three enzymes catalyse ring deaminations (aminohydrolases: EC 3.5.4.–), a known reaction type seen here with novel substrates. The second three enzymes appear to be ureases (amidohydrolases: EC 3.5.2.– and EC 3.5.1.–). These types of catalysis are also known [125,126], but this particular ring cleavage is novel, and these results are the first proof of the nature of the 'biuretase' reaction [79,127], whereas the urease reaction (EC 3.5.1.5) is well-known [125].

There is a different enzyme for each individual reaction in AAAT degradation. Many reactions are specific, but at least one enzyme has a broad substrate specificity [20,79,89,90]. In strain A (Table 2), the AAAT aminohydrolase also quantitatively hydrolyses PAAT, CAAT and even OAAT. There is a separate OAAT aminohydrolase in strain A which quantitatively hydrolyses POAT (and many homologues) and possibly COAT: in contrast, the OAAT aminohydrolase from strain D does not hydrolyse homologues of POAT. The OOAT aminohydrolase from strain A, as well as the ring cleavage and urease reactions, appear to be highly substrate-specific. Biuretase is still difficult to study, because it is labile, but it too seems highly specific.

POOT is funnelled into the main degradative pathway by a reaction analogous to that catalysed by the ring aminohydrolases (EC 3.5.4.–). This alkylaminohydrolase cleaves the side-chain as the alkyl amine, and the *s*-triazine product is OOOT (Fig. 5) [20]. The alkylaminohydrolase seems to dealkylaminate only bis(hydroxy)-*s*-triazines, but it can accept long (C_4) and short (C_1) side-chains. This novel hydrolytic reaction occurs only in strain D (and the closely related strain F; see section *4.2.2.1*) and differs markedly from the oxidative cleavage of the alkyl group from the alkylamino side-chain (Fig. 4).

Chlorinated *s*-triazines are funnelled into the pathways described above by yet another novel enzyme(s) (Fig. 5). CEAT is subject to hydrolytic ring dechlorination to yield EOAT and chloride ion [91,128]. Two isofunctional enzymes dechlorinate both CIAT and CEAT but not the bis(alkylamino)derivatives (e.g., CIET) or the hydroxy analogues (e.g., CEOT, COAT or COOT). We presume a similar reaction type to be involved in

the dechlorination of COOT to OOOT (Fig. 5; Table 2) in bacterial strain C3E.

s-Triazine catabolism in these organisms thus forms a previously unknown set of pathways converging at an intermediate that is subject to ring cleavage (Fig. 5). This is analogous to the pathways observed in degrading most other complex heterocycles [126] (cf. [129]) and in the well-known pathways of metabolism of benzenoids (e.g., [130]).

6.3. Other reactions

The reactions defined in Fig. 5 substantiate and expand several of our earlier reports and ideas [88,117] but do not represent a complete picture of the variety of *s*-triazine metabolism. Whereas we now [92] suspect a larger series of converging catabolic pathways (Fig. 6) resulting from hydrolytic reactions at the *s*-triazine nucleus, Giardini's group [83,84,131] has evidence of a totally different mechanism of removal of a substituent from the *s*-triazine ring (Fig. 4B). A product (presumably CAAT) from oxidative dealkylation of CIET is subject to a reaction that liberates a hydrotriazine (CAHT, Fig. 4B) [83]. The mechanism is unknown, and difficulties with the organism (sections *5.1.2* and *5.1.4*) are compounded by the properties of CAHT, which, typically of a hydrotriazine [126], hydrolyses spontaneously in aqueous solution. Barnes and Eagon [87], working with a saturated *s*-triazine, are uncertain whether degradation (presumably hydrolysis) to putative formaldehyde and ethylamine is spontaneous or biological or both. A likely source of new degradative enzymes is the work of Kaplan's group, which has good analytical data for the degradation of the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine in anaerobic sewage [86].

6.4. Summing up

The best understood reactions in *s*-triazine degradation are the hydrolytic reactions shown in Fig. 5. There is a simple reason for this, the enzymes have high specific activities (Table 5), they are easily assayed, and they can be produced in relatively large amounts. These conditions, so conducive to biochemical research, do not hold for the other areas of *s*-triazine metabolism.

Few of the hydrolytic reaction types are novel. Nevertheless, degradative pathways consisting solely of six successive hydrolases are unusual

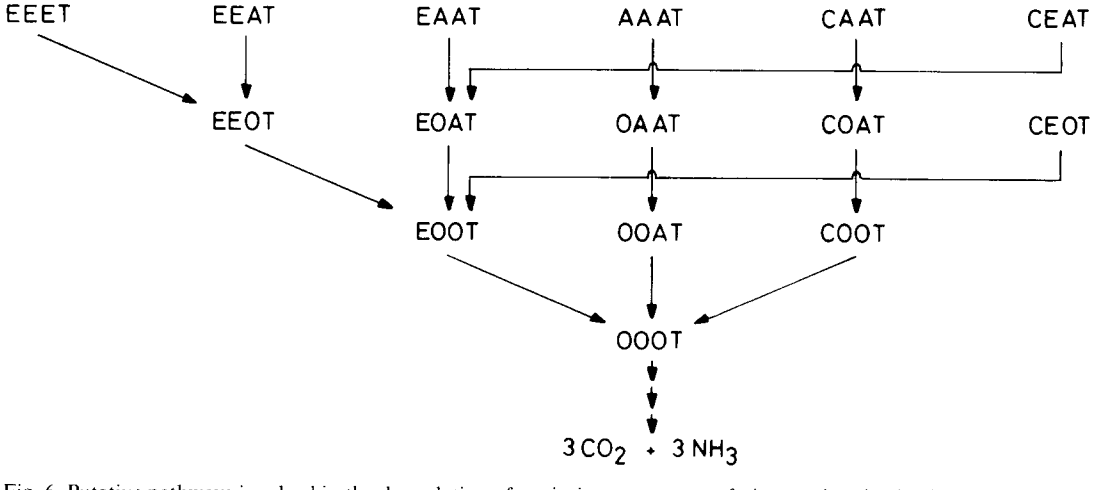

Fig. 6. Putative pathways involved in the degradation of *s*-triazines as sources of nitrogen in mixed cultures utilising *s*-triazine wastes. In this diagram, the ethyl amino group is used as an example, but this represents any alkyl amino group with a chain length of C1 to C4. The reactions represent an extension of the data in Fig. 5, and each intermediate shown was tentatively identified by HPLC [92]. Pathways via *N*-dealkylation are possible, but the appropriate intermediate was never observed; furthermore, the culture was developed by mutation from strain D (and similar isolates, though with other organisms under non-sterile conditions) so we represent the reactions, where appropriate, as dealkylaminations, until more data are available.

[126]: even the anaerobic degradation of guanine involves two separate sets of three hydrolases each [132]. Furthermore, reports of hydrolytic dechlorinations at an aromatic ring are still rare [133,134] compared with occasional oxidative dehalogenation and the more frequent dehalogenation subsequent to ring cleavage [135], and with the reductive reactions [77,136]. The hydrolytic reactions at the aromatic *s*-triazine ring are facilitated by the incomplete delocalisation of the π-electrons [112], which results in a low electron density at the carbon atoms and susceptibility to nucleophilic attack.

We do not know how *s*-triazines enter the cell but we have two reasons for presuming that transport systems are necessary to allow cells to utilise the compounds. Firstly, there is indirect physiological evidence for the transport of the intermediate biuret (sections *5.2*, *5.3*). Secondly, bases such as uracil (an analogue of *s*-triazines) require transport systems to cross the cell membrane [137].

The regulation of synthesis of enzymes of *s*-triazine degradation is also little understood. Most enzymes studied seem to be synthesised constitutively (Table 5). This lack of regulation of converging catabolic pathways contrasts with the situation in the benzenoid system, where enzyme synthesis is subject to careful control [138]. One reason for this difference in regulation may lie in the fact that all enzymes for any one degradative pathway are seldom located in any one organism, and a consortium is required for full degradation; another reason may lie in the fact that the *s*-triazines supply nitrogen for growth and no energy (except perhaps from alkyl side-chains) whereas the degradation of (nitrogen-free) benzenoid aromatics supplies carbon and energy for growth. Alternatively, the enzymes may be newly evolved, which normally requires a regulatory mutation, and the regulation of enzyme synthesis may be incompletely evolved [139].

7. EVALUATION OF MICROBIOLOGICAL ASPECTS OF THE BIOTREATMENT OF TRIAZINE WASTES

One aspect of our project was to create a microbiological treatment for recalcitrant xenobiotic compounds (*s*-triazines) and thus to learn how better to attack this type of problem. The *s*-triazines in the wastewater are now biodegradable (section *2*) and the cultures we obtained can indeed treat real wastes [23,40]. The treatment turns out to be impractical for three reasons: the low specific activity of some degradative reactions; the cost of the carbon source for growth; and the inadequate salt tolerance of the cultures [40]. These reasons may appear trivial, but they represent part of the learning process from what we believe to be the first attempt to create a defined biological treatment system specific for one multi-component, xenobiotic waste-stream. The question is how these deficiencies occurred and how to avoid or overcome them.

7.1. Model waste

The project was originally basic research on the biodegradability of *s*-triazines in (model) wastewater. The decision to do nitrogen-limited enrichments (section *4.2.2.1*) led directly to organisms proven to degrade *s*-triazines (section *5.1.2*) in the model waste. The specific degradation rates of about 0.4 mkat/kg of protein (section *5.2*) were important because they were considered high enough, allowing a concentration factor of about 10 for immobilising cells, to attempt to treat real wastes: Munnecke operated a treatment for the insecticide parathion at about 4 mkat/kg of protein [140]. We anticipated treating wastes with non-growing cells in some form of non-sterile bioreactor which was directly connected to the undiluted waste stream from the chemical synthesis [40].

7.2. Real waste

The project was declared applied research, and we received real wastewater, which we found to have almost nothing in common with the model waste [13]. The enzymes we had examined were mostly specific (section *6.2*), so we had enrichments for the wrong degradative abilities (section *4.2.2.1*). Instead of applying our original bacteria to treat wastes, we had to restart enrichment cultures, and some of the degradative cultures we

obtained (section *4.2.2.2*) had low specific activities [23].

Two phenomena can be recognised here: (a) the specificity and the specific activities of the bacteria; and (b) the difference between model waste and real waste.

Firstly, the strength of this biodegradation project is that specifically identified, recalcitrant xenobiotics can be examined individually, and degradative organisms can be obtained. Also, the specific activity of an enzyme can usually be increased, if necessary [141]. If, however, the wrong component is chosen for study, the wrong organism will be obtained.

Secondly, the model waste turned out to represent an outdated production method (section *3*). Analyses of the real wastewater were indeed available, but they were based on several old techniques [17] and they missed several components, which we discovered [13]. Estimates of the contents of wastewater, and far less the global parameters, e.g., total organic carbon [100], are totally inadequate bases for the specific biodegradation we attempt. A project of this kind can only succeed when the compounds to be degraded, as well as the physiological conditions (see next paragraph), are specified prior to starting enrichments, and when these compounds are made available, pure, in large amounts.

Two other problems in the treatment of real waste were the cost of a carbon source and the cost of diluting the wastewater because of the poor salt tolerance of our cultures. We had anticipated the use of non-growing cells (section *7.1*), which would be grown up occasionally and whose *s*-triazine-degrading enzymes would be largely resistant to the effectively marine environment [142]. We thus chose carbon sources most likely to facilitate growth of the nitrogen-limited enrichment cultures. The problems became clear when we observed that only growing cells could be used to treat wastes [23]. It is now obvious that integrated waste treatment should have been built into the project by choosing a waste carbon source(s) for enrichment cultures, and that the inorganic portion of the growth medium for enrichment cultures should have contained sodium chloride (see also [143,144]).

Thus, factors, which, if recognised, could easily have been considered when planning enrichment cultures, combined to make our treatment system impractical. This illustrates that details of any planned application of biodegradation must be known in advance so that the correct aims for the enrichment cultures can be set.

Future projects should have a further option available to them, (molecular) genetics. Our project would then require research on increased specific activities for some enzymes, degradation of a suitable carbon source or sources, and on suitable salt tolerance. Given suitable recipients, the genes coding for the various properties could be transferred to create organisms tailored to a particular problem: any attempt to treat a spill of formulated pesticide would have to treat pesticide-containing formulating agents, as opposed to the chemically more homogeneous and pesticide-free by-products examined in our work.

But is this feasible? Biological waste treatment (the activated sludge process) may be less expensive than chemical or physical treatments [145] but is a waste-specific bioreactor (section *7.1*) also less expensive? Will degradative strains be used only to seed activated sludge plants or contaminated soil [143,144,146,147], or will, e.g., analytical aims be served [148] (cf. [147])? Will our industrial history of poorly controlled waste dumps and abused rivers force authorities to initiate large, long-term biodegradation projects as one aspect of efforts to clean up badly contaminated sites? The need is there.

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