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Microbial metabolism of sulfur- and phosphorus-containing xenobiotics

M.A. Kertesz *, A.M. Cook and T. Leisinger

Institute of Microbiology, Swiss Federal Institute of Technology, ETH-Zentrum, CH-8092 Zürich, Switzerland

Abstract: The enzymes involved in the microbial metabolism of many important phosphorus- or sulfur-containing xenobiotics, including organophosphate insecticides and precursors to organosulfate and organosulfonate detergents and dyestuffs have been characterized. In several instances their genes have been cloned and analysed. For phosphonate xenobiotics, the enzyme system responsible for the cleavage of the carbon–phosphorus bond has not yet been observed in vitro, though much is understood on a genetic level about phosphonate degradation. Phosphonate metabolism is regulated as part of the Pho regulon, under phosphate starvation control. For organophosphorothionate pesticides the situation is not so clear, and the mode of regulation appears to depend on whether the compounds are utilized to provide phosphorus, carbon or sulfur for cell growth. The same is true for organosulfonate metabolism, where different (and differently regulated) enzymatic pathways are involved in the utilization of sulfonates as carbon and as sulfur sources, respectively. Observations at the protein level in a number of bacteria suggest that a regulatory system is present which responds to sulfate limitation and controls the synthesis of proteins involved in providing sulfur to the cell and which may reveal analogies between the regulation of phosphorus and sulfur metabolism.

Key words: Xenobiotics; Biodegradation; Phosphorus metabolism; Sulfur metabolism; Global regulation

Introduction

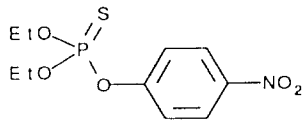
Xenobiotic compounds have been defined as "compounds that are released in any compartment of the environment by the action of man and therefore occur in a concentration in this or another compartment of the environment that is higher than 'natural'" [1]. Most often, these compounds are chemicals whose synthetic nature and non-natural structure preclude or retard their degradation by microbial species, and therefore lead to their accumulation in the environment. However, even the most persistent xenobiotic compounds can be metabolized to some extent by bacterial cultures [2], either by co-metabolism with other substrates [3,4] or during their utilization as sources of energy or nutrients (carbon, nitrogen, phosphorus or sulfur). As this review will show, the mode and extent of degradation of a xenobiotic compound by any particular organism depends crucially on which of these elemental components is required by that organism for growth.

The metabolic pathways involved in assimilation of the above nutrients by bacteria are regulated not only by specific substrate induction mechanisms, but to a large extent also by global control systems [5]. Catabolite repression exerts global control on utilization of various sources of carbon; in the presence of a 'preferred' carbon

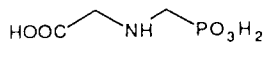
* Corresponding author.

source, expression of the genes involved in metabolism of alternative compounds are repressed [6,7]. Global nitrogen regulation (the Ntr system) likewise regulates expression of a large number of different genes, in response to ammonia levels within the cell [6]. A third mode of global cellular regulation is the stringent response, which reacts to starvation for either amino acids or carbon and energy sources, and is mediated by levels of the nucleotide ppGpp [8,9]. Global systems for regulation of phosphorus metabolism have also been well characterized, and have been described in a recent review [10]. The Pho regulon is governed by a two-component sensor–regulator system which in enteric bacteria controls the expression of a number of genes concerned with uptake and metabolism of non-phosphate phosphorus. For sulfur, by contrast, relatively little has been reported. In *Pseudomonas putida*, *Staphylococcus aureus* or *Escherichia coli* a set of proteins is coordinately induced during growth with sulfur sources other than cysteine or sulfate [11]; this regulatory network was termed the SSI-stimulon (sulfate starvation-induced), due to the superficial resemblance of the response to the synthesis of phosphate starvation-induced (PSI) proteins during growth with alternative phosphorus sources. However, the mechanism of regulation and the nature of the factors involved are still unknown. For carbon, nitrogen and phosphorus metabolism, cross-talk between regulatory systems has also been observed, leading to a highly complex control network capable of finely tuned responses to environmental signals [12–15].

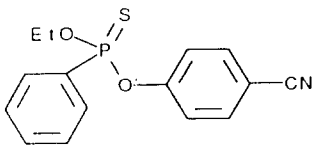
In most studies of xenobiotic degradation, the compounds under investigation have been supplied to microorganisms exclusively as sources of carbon and energy. Since bacteria require significantly more carbon for growth than any other nutrient, this leads to maximum removal of the xenobiotic, an important aim in studies concentrating on detoxification or bioremediation. Utilization of xenobiotics as sources of phosphorus and sulfur has been less well studied until now. In this review we will concentrate on the metabolism



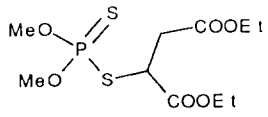
Parathion



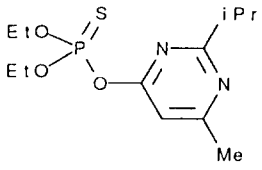
Glyphosate



Cyanofenfos



Malathion



Diazinon

Fig. 1. Some phosphorothionate (parathion, diazinon), phosphorodithionate (malathion) and phosphonate (glyphosate, cyanofenfos) xenobiotics. Glyphosate is a herbicide, and the other compounds are insecticides. Me, methyl; Et, ethyl; iPr, isopropyl.

of selected phosphorus- and sulfur-containing xenobiotics, and its control.

Phosphorus-containing xenobiotics

Phosphorus-containing xenobiotics are of great economic importance and have found extensive application in recent years. They are generally considered to be non-persistent, and a wide range of them can be broken down by bacteria [16]. The phosphorus is usually present in the molecule either as a phosphate ester or as a phosphonate (Fig. 1). Organophosphates act as inhibitors of acetylcholinesterase in the nervous system and have found application as insecticides in agriculture and in control of insect-borne diseases. The most widely employed of these compounds are the phosphorothionate parathion and the phosphorodithionate malathion (Fig. 1). Organophosphonates possess antibacterial, antiviral and antitumour activity, and are also used as herbicides, as detergent additives and as flame retardants [17]. They contain a direct carbon–phosphorus linkage, which is chemically and thermally very stable and renders the molecule much more resistant to non-biological degradation in the environment than its analogues with N-P, S-P or O-P linkages [18]. The xenobiotic character of organophosphonates is also emphasized by the relative rarity in nature of compounds bearing a C-P linkage [17]. The most widespread phosphonate xenobiotic is *N*-phosphonomethylglycine, better known as glyphosate, or under its tradename of Roundup (for a review, see [19]). It is a broad spectrum, post-emergent herbicide acting against virtually all annual and perennial plants, primarily by disrupting the biosynthesis of aromatic amino acids [20].

Phosphonate xenobiotics – glyphosate

Glyphosate is rapidly degraded in the environment, and a number of bacterial species have been isolated which can break down the compound. These include a *Flavobacterium* species [21], several *Pseudomonas* species [22–25] (of which the best studied is *Pseudomonas* sp. PG2982 [23]), an *Alcaligenes* isolate [24], *Bacillus megaterium* strain 2BLW [22], *Arthrobacter* sp. GLP-1 [26], four different *Rhizobium* species [27] and three *Agrobacterium* species [27,28]. Biodeg-

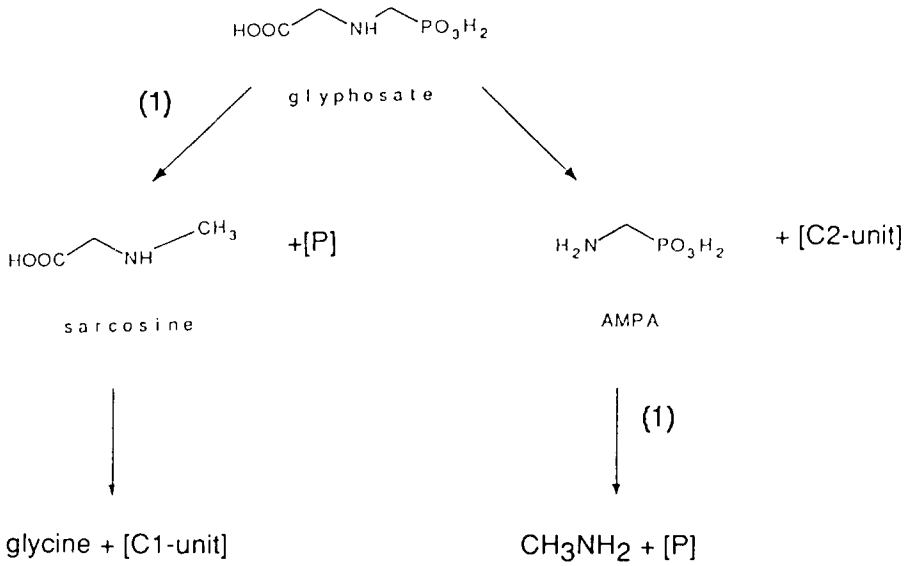


Fig. 2. Degradative pathways for glyphosate. Reactions (1) are catalysed in vivo by a 'C-P-lyase'. Phosphorus is utilized by the cell as inorganic phosphate. The initial phosphorus product of the lyase reaction is unknown, as are the one-carbon and two-carbon cleavage products. AMPA stands for aminomethylphosphonic acid.

radation has thus been found in both Gram-positive and Gram-negative organisms, but not in eukaryotes or cyanobacteria.

Glyphosate is degraded in bacteria by two main pathways, both of which lead to breaking of the carbon–phosphorus bond. In the first of these, initial cleavage of the carbon–phosphorus bond yields the reduced product *N*-methylglycine (sarcosine). This pathway has been found in *Arthrobacter* sp. GLP-1 [26] and in *Pseudomonas* sp. PG2982 [29]. The sarcosine formed has been shown by $^{13}C/^{15}N$-NMR studies to be further degraded to glycine and a C_1-unit, which is incorporated into purines and the amino acids serine, cysteine, methionine and histidine [26]. Alternatively, glyphosate may first be converted to aminomethylphosphonic acid (AMPA) by loss of a C_2-unit, and this compound is then dephosphonylated (e.g. in *Arthrobacter atrocyaneus* [30] or in *Flavobacterium* sp. [21]) (Fig. 2).

The enzyme or enzymes responsible for the key dephosphonylation step are known by the general name 'C-P lyase'. Despite extensive efforts to stabilize them [23,26,28,31,32], C-P lyase activity measured as alkane release from alkanephosphonates has never been observed in cell-free extracts. It has generally been assumed that the mechanisms involved in cleavage of the various organophosphonates are similar, although recent evidence shows that, in *Arthrobacter* sp. GLP-1, glyphosate and methanephosphonate are probably degraded by distinct enzymes, based on differential inhibition of sarcosine or methane production by substrate analogues [31]. This conclusion is supported by the fact that several species which possess C-P lyase activity and can cleave alkanephosphonates efficiently, including *E. coli*, cannot metabolise glyphosate at all, even when the growth medium is suitably supplemented with aromatic amino acids.

Despite the relative rarity of the C-P bond in nature, the ability to degrade glyphosate as a sole source of phosphorus for growth appears to be common in the environment [33]. Although their natural role is unknown, the enzymes of glyphosate metabolism were presumably present in the environment before the herbicide came on the market in 1971, since a strain of *Arthrobacter atrocyaneus* which was deposited in a culture collection prior to the introduction of the herbicide can also metabolize the compound [30]. In almost all studies of glyphosate degradation, the herbicide was supplied solely as a source of phosphorus, and the organisms investigated were not able to use it as a source of carbon or nitrogen. Although a mutant of *Arthrobacter* sp. GLP-1 which was capable of utilizing both the nitrogen and the phosphorus of glyphosate has been reported, it grew extremely slowly and proved on analysis to have a defective phosphate transport system [34]. Similarly, when glyphosate was supplied as a source of carbon to a microbial consortium in a bioreactor, full mineralization was not observed; cleavage of the C-P bond did not occur, and the main product found was AMPA [35].

C-P lyase has been most extensively investigated by assaying the release of methane from methanephosphonic acid by whole cells of *E. coli*. The details of the reaction mechanism are largely not understood, although the reaction is known to constitute direct reductive cleavage of the phosphonate without prior activation [36], and may involve radical intermediates [36–39]. However, further biochemical investigations have been hindered by the lack of cell-free C-P lyase activity, and recent studies have concentrated on the genes involved in phosphonate degradation. By introducing Mud1-mediated *lacZ* insertions into the genome of *E. coli* a series of phosphate starvation-induced loci was identified [40]. In one of these (*psiD*), the insertional mutants also lost the ability to utilize phosphonates [41]. Complementation of these mutants using the mini-Mu in vivo cloning procedure [42] has led to the identification of the genes presumed to code for phosphonate uptake and degradation. Seventeen open reading frames were found in the *psiD* region (now renamed *phnA* to *phnQ*) [43], of which 14 (*phnC* to *phnP*) were shown to be transcribed as part of a 10.9-kb operon [44,45]. Transcription was found to begin at *phnC*, where a *pho* box was located and a possible transcription start site identified [46]. On the basis of sequence analysis and comparison with known motifs, reading frames *phnCDE* have been proposed to form a phosphonate transport complex, whereas *phnF*

and *phnO* may be involved in regulation, since they are not absolutely required for phosphonate utilization [45]. It is interesting that attempts to broaden the specificity of phosphonate utilization in *E. coli* have led to mutations only in the *phnCDE* genes [45], suggesting that uptake may be the limiting factor in this respect. The remaining genes may be involved in the C-P lyase complex itself, which would then include some nine gene products, some of which were deduced from the sequence to be integral membrane proteins [44]. In this case, the C-P lyase may well be a large, and fragile, membrane-associated protein complex, and difficulties experienced in obtaining cell-free activity could be due to its unstable nature.

Metcalf and Wanner [44] also observed that mutants losing the ability to utilize phosphonates as sole source of phosphorus simultaneously lose the ability to assimilate phosphite; complementation of the one phenotype leads without exception to restoration of the other as well. This has led to the proposal that phosphonate degradation by C-P lyase proceeds reductively to yield phosphite (either free or enzyme-bound) as the initial phosphorus product of the reaction, followed by phosphite oxidation to inorganic phosphate [45]. This constitutes the first evidence for a redox cycle for phosphorus in nature, comparable to that observed for sulfur [44].

Phosphorothionate insecticides – parathion and malathion

The ability to degrade phosphorothionate and phosphorodithionate insecticides also appears to be widespread in the environment, and a variety of bacterial species have been isolated which can utilize these compounds (for reviews, see refs. [47,48]). These include species of *Flavobacterium* [49], *Arthrobacter* [50], and *Pseudomonas* [51–54], as well as many isolates which were not further identified [55–57]. In principle, parathion and malathion may also provide both sulfur and phosphorus for growth, but research into this aspect of organophosphate degradation has been much less extensive.

The initial step in bacterial parathion degradation is the hydrolysis of the arylphosphate bond, giving rise to diethylphosphorothionate and *p*-nitrophenol (Fig. 3). These products are the same whether the parathion is used as a carbon source

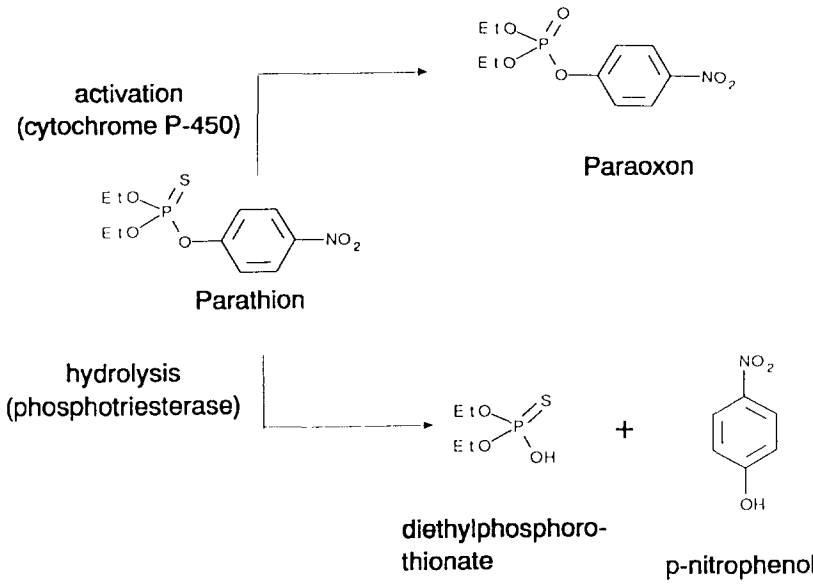


Fig. 3. Activation and hydrolysis of parathion. Since paraoxon is the metabolite responsible for parathion's neurotoxic effect, the relative toxicity of parathion observed in different organisms is determined by the balance between the two pathways.

[55,57,58] or to provide phosphorus for growth [59]. The enzyme that catalyses the hydrolysis is a phosphotriesterase (aryldialkylphosphatase, EC 3.1.8.1). In insects and other higher organisms, parathion is rapidly converted by monooxygenases to its phosphate homologue paraoxon, which is responsible for the toxic effect of the pesticide. Paraoxon is also toxic to humans, but parathion itself is less so, since humans possess efficient esterase systems which hydrolyse the arylphosphate bond before paraoxon can accumulate. The relative toxicity of the compounds to different species therefore depends on a balance between oxidative activation of parathion to paraoxon and esterase-mediated deactivation [60] (Fig. 3). However, in bacteria this oxidative reaction constitutes only a minor pathway [58]. The phosphotriesterase has been purified from *P. diminuta* MG [61], *Flavobacterium* sp. ATCC 27551 [62], and from two unidentified strains, B-1 and SC [56]. In the former two organisms, it is a 35-kDa, membrane-associated enzyme, and in *P. diminuta* it appears to exist as a dimer [61]. Phosphotriesterase in strain B-1 was found to be a cytosolic, 43-kDa enzyme, while in strain SC the enzyme consisted of four identical 67 kDa subunits [56]. Phosphotriesterase catalyses a single hydrolysis of arylphosphotriesters to the dialkyl ester products, and accepts a broad range of aryldialkylphosphotriesters as substrate [57,63], including both phosphate esters and phosphorothionates [57].

The phosphotriesterase enzymes found in *P. diminuta* and *Flavobacterium* sp. ATCC 27551 have both been shown to be encoded on large plasmids, pCMS1 (51 kb) and pPDL2 (39 kb)

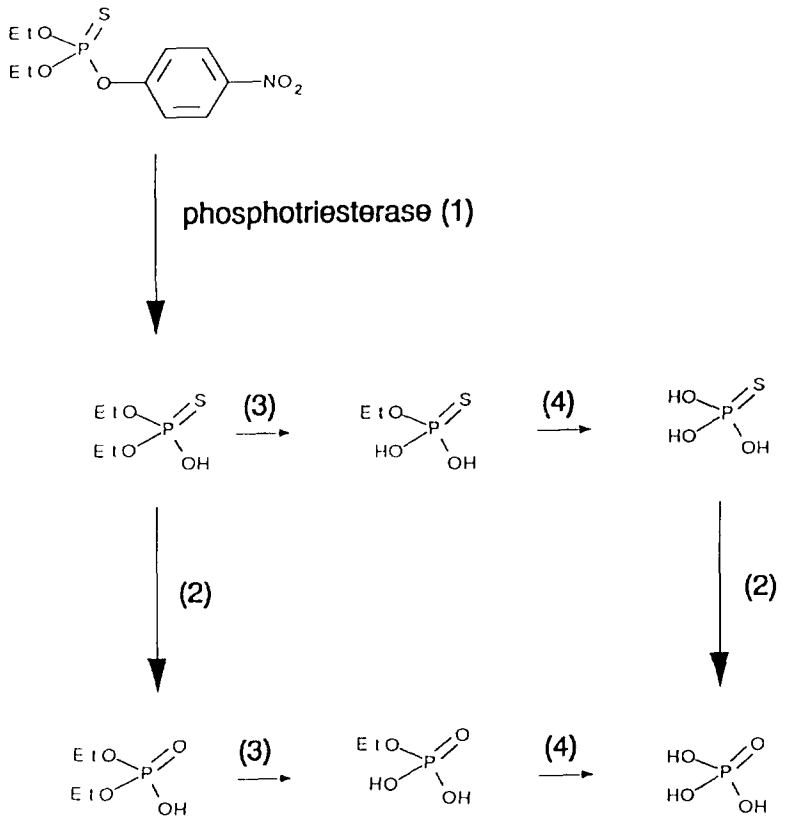


Fig. 4. Hypothetical further degradation pathways for diethyl phosphorothionate. Reactions (2) represent either chemical or enzymic oxidations, reactions (3) are catalysed by a phosphodiesterase, and reactions (4) by a phosphomonoesterase (e.g. alkaline phosphatase).

(also called pSM55; 43 kb [64]), respectively [61,62]. The two *opd* (organophosphate degradation) genes have been cloned and sequenced [61,62,65], and proved to be 100% identical [64], although restriction mapping confirmed that the plasmids carrying them differed extensively [64]. Both of these enzymes are produced constitutively, and have usually been purified from nutrient broth cultures grown without addition of phosphotriesters as inducers.

Degradation of parathion to *p*-nitrophenol and diethylphosphorothionate leads to a reduction in the mammalian toxicity by a factor of 122 [47]. Since most interest has been directed towards detoxification, studies on the further metabolism of the phosphorus-containing products have not been extensive. Further hypothetical phosphoester hydrolysis steps can be postulated, yielding the monoester and finally inorganic phosphate (Fig. 4), but this pathway has not been specifically studied. Analogous phosphomonoesterases and phosphodiesterases which degrade methyl and dimethyl phosphate, respectively, have been reported in *Klebsiella aerogenes* [66], and are produced only in the absence of inorganic phosphate from the growth medium. The final enzyme in the postulated degradative pathway (Fig. 4), bacterial alkaline phosphatase, is well-studied and has been the subject of recent reviews [67,68]. This enzyme can hydrolyze simple monoalkyl phosphates [66], and is also regulated by the levels of phosphate available to the cell.

The products of phosphorothionate hydrolysis have also been investigated as sources of sulfur and phosphorus for bacterial growth. The way in which their metabolism is regulated depends very strongly on what role the phosphorothionate plays for the particular organism studied. Most often the compound is used to supply only a single element (carbon, phosphorus or sulfur), and the relevant genes cannot be expressed as a response to starvation for another of these elements. For example, it has been shown that ionic dialkylphosphates and their sulfur analogues can be utilized as phosphorus sources by environmental isolates [69], but with one exception the strains isolated in this study were unable to utilize the compounds as a sole source of sulfur for growth [70]. A strain of *Pseudomonas stutzeri* isolated to utilize parathion as a carbon source released the diethylphosphorothionate product quantitatively, and could not metabolize it further even when alternative sources of phosphorus or sulfur were removed [51]. Correspondingly, a variety of isolates which could use phosphorothionate and phosphorodithionate pesticides as a sole source of phosphorus [59] were unable to degrade these as a source of carbon and energy for growth. Clearly, the conditions under which environmental isolates are enriched in the laboratory are crucial in selecting for strains not only with the desired degradative enzyme systems, but also with specific regulation mechanisms for the biodegradation pathways.

Phosphate-mediated control of gene expression

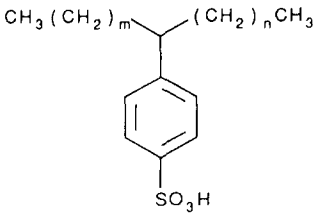
The best characterized system for phosphate control of enzyme expression is the Pho regulon (for recent reviews see [10,71]). In *E. coli* this regulon comprises some 81 proteins whose expression is increased during phosphate deprivation [72], the best characterized of which is alkaline phosphatase. The *phn* (*psiD*) (phosphonate degradation) locus is also regulated as part of this regulon. C-P lyase activity was measured in vitro during growth with limiting phosphate (60 μM) and methanephosphonic acid as phosphorus sources [41]. As the inorganic phosphate was exhausted, a pause in growth was observed, and both alkaline phosphatase and C-P lyase (measured as methane generation) were then induced simultaneously and in parallel [41]. A very similar result was obtained for *Agrobacterium radiobacter* during growth with ethanephosphonate and inorganic phosphate (each 50 μM) [28]. The addition of inorganic phosphate (100–500 μM) to growing cultures of *A. radiobacter* led to a cessation of methane production [28], although this effect might also have been due to inhibition of phosphonate transport. Glyphosate transport in *Arthrobacter* sp. GLP-1, for example, is also inhibited by phosphate [73]. Uptake of glyphosate into the cell is subject to both inhibition and repression by phosphate in this species [73], and phosphate starvation also leads to derepression

of glyphosate transport in *Pseudomonas* sp. PG2982 [74].

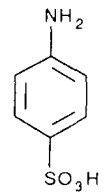
The evidence for phosphate-mediated control of phosphorothionate and phosphorodithionate degradation is less conclusive. In *Pseudomonas* species isolated for their ability to degrade phosphorothionates as a source of phosphorus, no phosphotriesterase activity was observed in cells which had been grown with inorganic phosphate [59]. This was interpreted by the authors as evidence that the enzyme must be purely substrate-induced, but the data are also consistent with Pho-control, which was not tested. Strains selected for utilization of ionic phosphorothionates as a phosphorus source were unable to degrade diethylphosphorothionate or diethylphosphorodithionate as a source of carbon [69], although they grew well with ethanol, the hydrolysis product of ionic phosphorodithionates. When the phosphorothionate is supplied as a carbon source, the relative cellular requirements for carbon and phosphorus may be expected to lead to release of a large excess of phosphate; it thus appears that phosphate levels may also be important in regulation in these isolates. The final step in the postulated parathion degradative pathway (Fig. 4), hydrolysis of a phosphate monoester by bacterial alkaline phosphatase, is also subject to control by inorganic phosphate as part of the Pho regulon [67,68].

Sulfur-containing xenobiotics

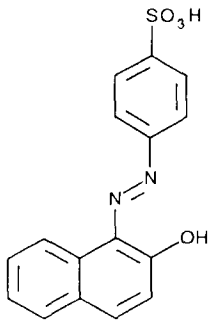
Sulfur in xenobiotic compounds is most commonly encountered in the form of sulfonate and



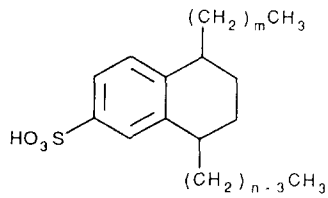
Linear alkylbenzene-sulfonate (LAS)



p-aminobenzene-sulfonate



Orange II



Dialkyltetralin-sulfonate (DATS)

Fig. 5. Representative arylsulfonate xenobiotics. The length of the alkyl chain in commercial LAS is C_8 to C_{20}. Dialkyltetralinsulfonate (DATS) is a byproduct in LAS manufacture, Orange II is a commercial textile dye, and *p*-aminobenzenesulfonate is an important intermediate in manufacture of drugs and of dyestuffs.

sulfate groups, bound either to an aromatic nucleus, as in the linear alkylbenzene sulfonate surfactants or the sulfonate dyes (Fig. 5), or to an aliphatic chain, as found in the alkyl sulfate detergents. The organosulfate or organosulfonate group plays an important role in altering the solubility and dispersion properties of the xenobiotic molecule, and in increasing its stability to environmental breakdown [75]. Sulfur also enters the environment in the form of thiourea, thiocarbamate and phosphorothionate agrochemicals, thioamide and sulfonamide drugs, thiophene derivatives from oil and coal combustion, and sulfur-containing heterocycles [76]. The current article will concentrate on the degradation of organosulfate and organosulfonate xenobiotics and its regulation; a broader survey is given in a recent review [76].

Because of their better surfactant properties and lower price, organosulfonate detergents have largely replaced traditional soaps and detergents, and by 1991 the linear alkylbenzenesulfonates (LAS) made up about 45% of the total surfactants used in the United States and Western Europe [77]. Most studies on their biodegradation have focused on primary biodegradation and removal of the surfactant nature of the molecule. Commercial LAS is an ill-defined mixture of isomers, with alkyl chain lengths ranging from C_8-C_{20} and the phenylsulfonate substituent located at various positions along the chain. However, most LAS compounds are now generally considered to be completely mineralized in nature [78].

Alkylsulfate detergents are also in common use, primarily in special laundry applications, but also in products as varied as toothpastes, antacids, insecticide emulsifiers or car cleaning shampoos [79]. The range of compounds on the market includes both primary and secondary alkylsulfates, and most are generally considered to be non-toxic and readily biodegradable, both in terms of primary biodegradation and complete mineralization [79]. However, the extent of biodegradability depends on the structure of the alkyl chain, and some of the branched, secondary alkylsulfates have been found to be highly resistant to degradation [78].

Aromatic sulfate esters are not in extensive commercial use, possibly because of the availability of the more stable aromatic sulfonate analogues. The enzymes responsible for their cleavage (arylsulfatases) are more easily assayed than for the alkylsulfates, since the aromatic phenol product can usually be quantified by spectroscopic methods. This represents a great contrast to the alkylsulfatases, where enzyme activity has usually been monitored as production of inorganic sulfate, an insensitive procedure until re-

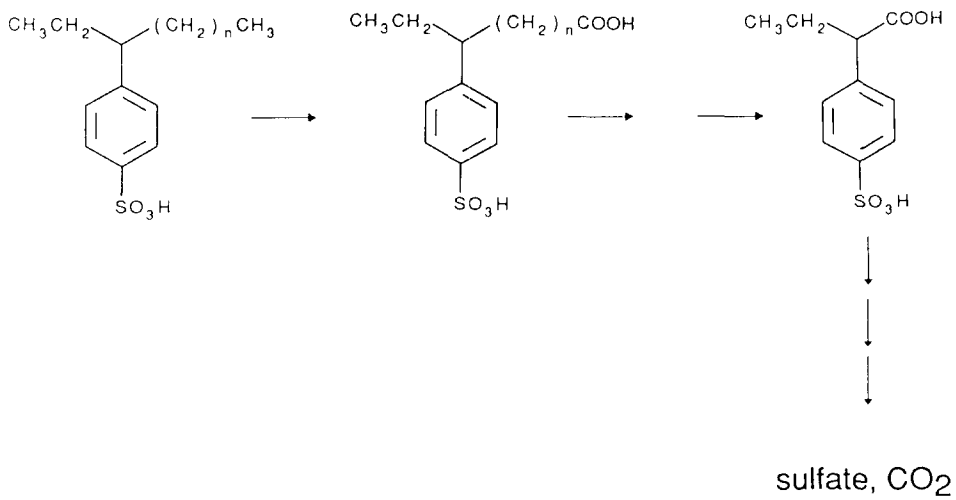
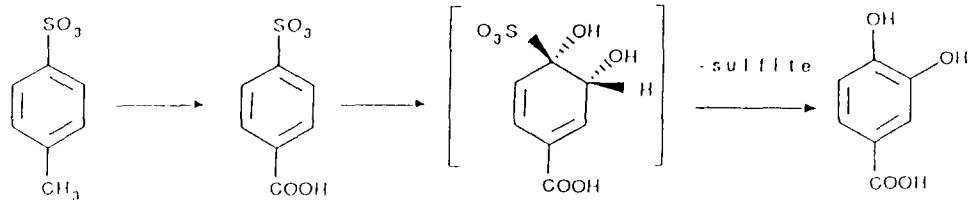
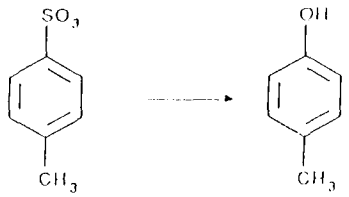


Fig. 6. Degradation of LAS. Terminal oxidation of the alkyl chain is followed by a series of β-oxidation steps. The order of desulfonation and ring cleavage in the final steps is not yet clear.

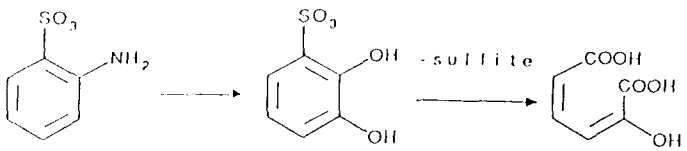
a) desulfonation prior to ring cleavage (85)



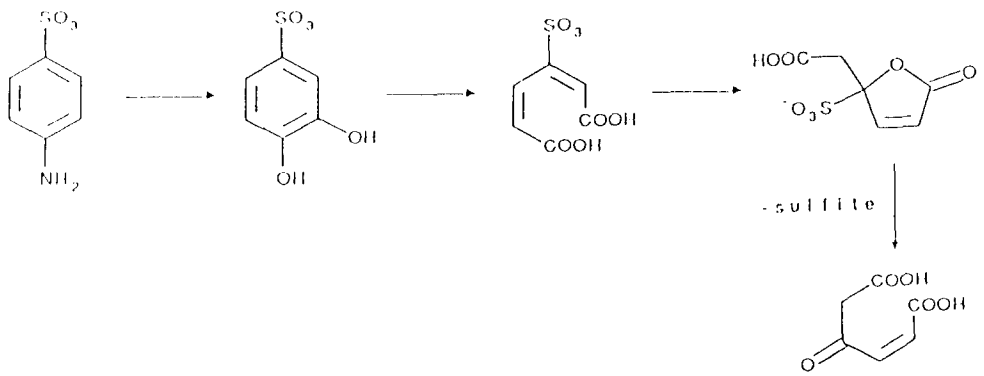
b) desulfonation prior to ring cleavage (91-93)



c) desulfonation concurrent with ring cleavage (94)



d) desulfonation after ring cleavage (95, 96)



cently [80–82]. Alkyl sulfate esters have commonly been supplied to the cells as a source of carbon and energy, and not solely of sulfur, whereas aromatic sulfates have been investigated almost exclusively as a source of sulfur. Given that the former case is expected to produce an excess of inorganic sulfate, and the latter an aromatic residue, the difficulties in accurate sulfate determination may be seen as one explanation of the different approaches adopted. With the advent of the new measurement techniques, it is to be hoped that further studies of alkylsulfate utilization as a sulfur source for common, well characterized strains will be undertaken.

Organosulfonates – detergents and dyestuffs

Studies of LAS biodegradation, and of aromatic sulfonate degradation in general, have concentrated almost exclusively on utilization of the target compounds as sources of carbon and energy for growth. In most cases, especially for LAS, the methods used have been ones which are relevant to environmental processes and bioremediation, such as die-away tests, respirometric methods (measurements of biological oxygen demand, BOD) or the use of trickling filters to study the rate of biodegradation. The inocula used have often been heterogeneous, including river or lake water, activated sludge, and soil samples. The biochemistry and, in particular, the regulation of the degradation pathways concerned are not yet well characterized.

The degradative pathway for LAS has been reviewed by Cain [83], and more recently by Swisher [78] and Schöberl [84]. Degradation is initiated by ω-oxidation of the methyl group more distant from the aromatic ring (Fig. 6), catalyzed by a monooxygenase. The aliphatic carboxylate chain is then degraded by a series of β-oxidation steps, probably catalysed by the same enzymes as those acting in fatty acid oxidation. This gives rise to a sulfophenylalkanoate residue such as sulfophenylbutyrate as an intermediate product. The aromatic sulfonate moiety, which lends the compound its xenobiotic nature [1], is at this stage still untouched.

Three modes of desulfonation are known for aromatic sulfonates: desulfonation (i) before, (ii) during or (iii) after ring cleavage. A well known type (i) mechanism involves oxygenation of the aromatic ring by a multicomponent oxygenase, yielding an unstable sulfono *cis*-diol which then spontaneously rearomatizes to the corresponding catechol with loss of sulfite (Fig. 7a). An enzyme of this type, catalysing degradation of *p*-sulfobenzoic acid to protocatechuic acid in *Comamonas testosteroni*, has been isolated and characterized [85], and the same mechanism has been found for desulfonation of benzenesulfonate and toluenesulfonate in an *Alcaligenes* sp. [86,87], and for naphthalenesulfonate metabolism in *Pseudomonas* and *Moraxella* species [88–90]. In all these cases, the sulfonates were provided as a carbon and energy source for the organism studied. A type (i) mechanism was also observed when arylsulfonates were supplied as a sole source of sulfur (Fig. 7b) [91,92]. In *Pseudomonas putida* S-313, a broad spectrum monooxygenolytic arylsulfonatase activity catalyzes the conversion of the sulfonate to a phenol with incorporation of one oxygen atom from molecular oxygen. The enzyme concerned has not yet been purified. Under the same conditions, *P. putida* S-313 can also desulfonate compounds related to LAS: *p*-sulfophenylbutyrate, *p*-octanesulfonate and dialkyltetralinsulfonate are each converted to the corresponding phenol [93]. No degradation of the sulfonate occurs in the presence of sulfate or cysteine, suggesting that the desulfonative system is subject to regulation by sulfur levels.

During degradation of *ortho*-aminobenzene sulfonate as a carbon source by *Alcaligenes* sp. O-1, desulfonation occurs by a type (ii) mecha-

Fig. 7. Four pathways of desulfonation of aromatic sulfonates. Desulfonation may occur prior to ring cleavage ((a) and (b)), concurrent with ring cleavage (c) or after opening of the aromatic ring (d). Pathways (a) and (c) were observed when the sulfonate was supplied as a carbon source for growth, pathway (b) when it provided only sulfur and (d) when it supplied carbon, nitrogen and sulfur.

nism [94], simultaneously with ring cleavage of the initial deamination product 3-sulfocatechol (Fig. 7c). This reaction presumably occurs via spontaneous decay of an unstable sulfonated intermediate. One example is also known of type (iii), desulfonation subsequent to ring opening (Fig. 7d). A syntrophic culture of *Hydrogenophaga palleroni* sp. S1 and *Agrobacterium radiobacter* sp. S2 metabolizes *p*-aminobenzenesulfonic acid via deamination to 4-sulfocatechol and meta-cleavage of the aromatic ring prior to loss of the sulfonate group [95,96]. Strain S2 metabolizes the sulfocatechol more efficiently, but only strain S1 can carry out the initial deamination reaction. The arylsulfonate was supplied as a sole source of carbon, nitrogen and sulfur for growth during the isolation process [96].

When simple alkanesulfonates are utilized as a carbon source by *Pseudomonas* species, the crude cell extract catalyzes oxidation of the α-carbon atom of the alkanesulfonate to an aldehyde bisulfite adduct. This adduct then decays to produce the corresponding aldehyde and sulfite. The substrate range is relatively broad, and hydroxy-, methyl- and alkenyl-substituted compounds are all transformed [97]. No studies of the regulation of this system have been carried out, nor has the enzyme concerned been purified.

Several of the enzyme systems involved in degradation of LAS and arylsulfonate degradation appear to be encoded on large degradative plasmids. In *Comamonas testosteroni* PtS1, genes for the LAS degradation pathway have been reported to reside on a 61-MDa plasmid [83]. There is also evidence that in *Alcaligenes* sp. O-1 the enzymes for degradation of 2-aminobenzenesulfonate are plasmid-encoded [98], as may be those for toluenesulfonate metabolism in *C. testosteroni* T-2 [99]. In general, it is considered that the LAS degradation pathways involved in carbon metabolism are inducible. Swisher [78] has reported that during growth with LAS as carbon source, a lag phase is observed between completion of side chain oxidation and the onset of ring degradation/desulfonation, suggesting that the required enzymes are directly substrate-induced. No sulfate repression effect on LAS degradation is observed in *Pseudomonas fluorescens* or *Aspergillus* sp. [100].

Organosulfates

Alkylsulfates – degradation and its regulation

The degradation of alkylsulfates proceeds by initial hydrolysis of the sulfate ester linkage, and subsequent oxidation of the released alkanol. This has been most extensively studied by the groups of Dodgson, Payne and Fitzgerald (reviewed in [101]). Microorganisms were isolated for their ability to utilize dodecylsulfate as a source of carbon and energy (but not of sulfur) for growth. This yielded *Pseudomonas* sp. strain $C_{12}B$ [102] and a strain of *Comamonas terrigena* [103]. Alternatively, enrichment with *n*-hexylsulfate as a source of carbon and of sulfur led to the isolation of a *P. aeruginosa* strain [104]. In *Pseudomonas* sp. $C_{12}B$, as many as five different alkylsulfatases are produced under various conditions. The characteristics of these enzymes, which have been titled P1, P2, S1, S2 and S3 (for *P*rimary and *S*econdary alkylsulfatases), and of the secondary alkylsulfatases CS1 and CS2 produced by *C. terri-*

Table 1
Expression of alkylsulfatases in *Pseudomonas* sp. $C_{12}B$ and *Comamonas terrigena* (101)

Enzyme	Organism	Substrate range	Regulation type
P1	*Pseudomonas* sp. C12B	Primary alkylsulfates, $C_{6,8,12}$	Constitutive
P2	*Pseudomonas* sp. C12B	Primary alkylsulfates, C_{4-14}	Inducible, catabolite repression
S1	*Pseudomonas* sp. C12B	(D)-2-alkylsulfates, C_{8-10}	Constitutive
S2	*Pseudomonas* sp. C12B	(L)-2-alkylsulfates, $C_{8,9}$	Constitutive
S3	*Pseudomonas* sp. C12B	(D)- and (L)-secondary alkylsulfates	Inducible, catabolite repression
CS1	*Comamonas terrigena*	(L)-2-octylsulfate	Constitutive
CS2	*Comamonas terrigena*	(D)-2-alkylsulfates, C_{6-14}	Constitutive

gena, have been described in detail [78,101]. The enzymes are completely specific for primary or secondary alkylsulfates, and are also very selective within these subclasses (Table 1). The S1, S2 and P1 enzymes each accept only a few particular organosulfates as substrate, whereas CS2 and P2 are rather less specific in this respect. The secondary alkylsulfatases even distinguish which isomer is accepted, the S1 and CS2 enzymes hydrolysing only substrates with D-configuration, while the S2 enzyme is specific for the L-form. All the alkylsulfatases cleave their substrates with inversion at the alcohol substituent, and hence with cleavage of the carbon–oxygen bond.

The synthesis of this family of enzymes has also been the subject of intense study. It is regulated in three main ways: constitutive synthesis, substrate induction, and derepression. P1, S1, S2, CS1 and CS2 are all produced constitutively. They are synthesized by the cells during growth in nutrient broth containing excess sources of both carbon and sulfur, and do not require the addition of an external inducer for expression [101]. Moreover, compounds which were found to repress the P2 and S3 enzymes, such as acetate, citrate or pyruvate, had no effect on synthesis of these constitutive sulfatases during growth in complex medium [101]. An early study [105] reported, however, that in minimal medium (1% citrate as carbon source, with 10 mM hexylsulfate as a possible enzyme inducer) none of the 'constitutive' enzymes was produced, so it is possible that an unidentified factor in the nutrient broth acts as an inducer in this case. In addition, there are distinct differences between the enzyme levels observed in exponential phase and stationary phase cultures, with variation as high as $\pm 70\%$ [101]. This suggests that the 'constitutive' synthesis reported for these enzymes is probably subject to more complex regulation than previously recognized.

The remaining two enzymes found in *Pseudomonas* sp. $C_{12}B$, P2 and S3, are not synthesized during growth in nutrient broth alone, but depend on specific induction by a suitable substrate. Sulfate esters (C_5–C_{14}) serve as excellent inducers of P2, but are rapidly hydrolysed by the enzyme. Comparable, though lower, levels of induction can be achieved with the corresponding alkylsulfonates (C_7–C_{12}) [106]. For S3, a combination of a long-chain secondary sulfate ester (1 mM) and its product alcohol is required for induction [107]. Production of P2 and S3 is also under negative control. Induction of P2 is prevented by primary alcohols (10 mM), by acetate (10 mM) or by succinate (20 mM), whereas synthesis of S3 is repressed by both short-chain primary and symmetrical secondary alcohols and by tricarboxylic acid cycle intermediates, suggesting that catabolite repression may be important in the regulation of both these enzymes.

The amount of sulfur source present in the growth medium plays no role in regulation of the *Pseudomonas* sp. $C_{12}B$ alkylsulfatases, although it is important in controlling the synthesis of sulfatases in other species. In a strain of *P. aeruginosa* isolated from soil for the ability to use hexylsulfate as a carbon and sulfur source, for example, there is evidence for control of alkylsulfatase by inorganic, sulfur-containing compounds (see below) [104]. However, even in this organism sulfur-controlled regulation is less important than the repression of enzyme synthesis which is observed in the presence of other carbon sources. Sulfur-mediated control essentially only modulates carbon-mediated regulation, since in the presence of tricarboxylic acid cycle intermediates, glucose, acetate or pyruvate (all 20 mM) no sulfatase was synthesized. These effects were in part concentration-dependent. Glucose, for instance, caused a stimulation of sulfatase expression at levels between 0.1 and 1.0 mM, but repressed severely at higher concentrations [104]. Nonetheless, the diversity of the carbon sources which repressed sulfatase synthesis [108] and the fact that reversal of succinate-mediated repression could be achieved by addition of 2,4-dinitrophenol (to reduce intracellular ATP levels) suggest that catabolite repression plays a role in regulation of this enzyme [108]. The complex regulation of this enzyme (substrate induction, catabolite repression and sulfate/cysteine-mediated repression simultaneously) may be partly due to its role in both anabolic and catabolic metabolism; the alkylsulfatase hydrolyses sulfate esters supplied as a source of carbon as part of a

catabolic process, but the same enzyme, by releasing sulfate, may also be regarded as catalysing the first step in the biosynthetic pathway leading to cysteine.

Arylsulfates

The arylsulfatases, which hydrolyse aromatic sulfate esters to the corresponding phenol and inorganic sulfate, are among the most common enzymes in natural soil environments [109–111]. Up to 50% of the total sulfur in soil is bound as ester sulfate, including a large proportion of arylsulfate esters derived from animal excreta (tyrosine sulfate, and sulfate esters formed in the detoxification of other phenols). Arylsulfates cannot, therefore, be regarded as true xenobiotics, but are included here because of the sulfur-controlled nature of arylsulfatase regulation. Unlike the alkylsulfatases, most of the arylsulfatases are not synthesized constitutively, but are subject to strict sulfur derepression control. They have been studied in various pseudomonads [101], in *Klebsiella aerogenes* [112–114], *Serratia marcescens* [115], *Salmonella typhimurium* [116], *Enterobacter aerogenes* [117,118], and in *Proteus rettgeri* [119]. These enzymes are also present in fungi (reviewed in [101]) and in higher organisms.

Regulation of arylsulfatase has been best studied in *K. aerogenes*, where it is subject to a complex system of controls (Table 2). Synthesis of the enzyme is repressed in the presence of sulfate, cysteine and closely related inorganic sulfur metabolites (3 mM) and derepressed by growth with methionine or taurine (3 mM) as sulfur source. In *K. aerogenes*, and several other enteric bacteria [101], the repressing effect of sulfate or cysteine can be overcome by addition of tyramine, dopamine or octopamine. These compounds are substrates and inducers for the enzyme monoamine oxidase. Measurements of arylsulfatase and monoamine oxidase activity demonstrate that the two enzymes are indeed synthesized concomitantly [120,121]. It therefore appears that expression of the oxidase gene is required for arylsulfatase derepression, although the reason for this is unclear. This requirement was confirmed using mutants unable to synthesize active oxidase [121], and others that did so constitutively [122] – in each case arylsulfatase synthesis was associated with that of the oxidase. Since expression of the monoamine oxidase gene is also subject to catabolite repression and to regulation by levels of ammonium and combined nitrogen [123,124], arylsulfatase expression in the presence of sulfate and tyramine is similarly regulated. *E. coli* contains a defective arylsulfatase gene, which has been studied by creating a fusion to a *lacZ* reporter gene [125], and by immunological studies with antibodies raised against arylsulfatase from *K. aerogenes* [118]. Expression of *atsA*, the *E. coli* arylsulfatase gene, is also in part controlled by monoamine oxidase [125]. However, the importance for the organism of this tyramine-mediated regulation of the sulfatase gene has yet to be clarified for either species.

Table 2
Regulation of arylsulfatase formation in *Klebsiella aerogenes* [112–114,121,126]

Effector	Presumed regulator or regulatory mechanism	Effect on enzyme specific activity
Sulfate	Unknown (cf. ssi)	Decrease
Cysteine	Unknown (cf. ssi)	Decrease
Unknown	*atsR* gene product	Decrease
Unknown	*atsB* gene product	Increase
Tyramine	Monoamine oxidase	Increase
Ammonia (+tyramine)	Ntr regulation	Decrease (by modulation of monoamine oxidase expresssion)
Succinate (+tyramine)	Catabolite repression	Decrease (by modulation of monoamine oxidase expresssion)

The arylsulfatase operon of *K. aerogenes* has recently been cloned [112], by complementation of a *K. aerogenes atsA* mutant. It is composed of two cistrons, the structural gene *atsA* and a putative positive regulator gene *atsB*. A negative regulator gene for arylsulfatase, *atsR*, has also been identified [126]. In *atsR* mutants, arylsulfatase is constitutively expressed, regardless of the sulfur source supplied. A gene complementing the *atsR* mutation was cloned and sequenced, and the corresponding amino acid sequence revealed 90.6% homology to that of the *E. coli* dihydrofolate reductase gene *folA* [126]. When the *atsR*

gene was introduced on a plasmid into either a wild type strain or an *atsR* mutant of *K. aerogenes*, an increase in dihydrofolate reductase activity of up to 30-fold was observed, demonstrating that *atsR* codes for a functional dihydrofolate reductase protein. However, *K. aerogenes* appears also to have a separate *folA* gene, since dihydrofolate reductase levels are very similar in the wild-type and the *atsR* mutant. The *folA* gene from *E. coli* was also found to complement the *atsR* mutation in *K. aerogenes* [126]. Why this connection between C_1-metabolism and arylsulfatase regulation exists and how it functions on a physiological level is not yet clear.

Enzyme derepression by sulfate deprivation

Arylsulfatases and alkylsulfatases

Regulation of enzyme synthesis by a sulfate starvation derepression mechanism was first studied in detail for the sulfur-mediated regulation of arylsulfatase in *P. aeruginosa* [127] and *K. aerogenes* [128]. Various compounds were supplied as the sole source of sulfur for growth, including sulfate, cysteine, methionine, several aromatic sulfate esters and inorganic sulfur sources such as sulfite and thiosulfate [127]. Expression of arylsulfatase was seen only in the absence of sulfate, cysteine, or closely related metabolites such as thiosulfate or sulfite. The same effect has since been observed in several other bacterial species, and is especially pronounced in fungi and yeasts [101,129]. For the bacterial species studied, methionine acts as a derepressing substrate, whereas in fungi methionine represses arylsulfatase expression [101]. The alkylsulfatase observed in the *P. aeruginosa* strain described above [104] is repressed by the addition of sulfate (39% reduction in enzyme activity), sulfite (27% reduction), sulfide (84% reduction) and cysteine (86% reduction).

Other proteins under sulfate control

A similar regulatory mechanism has also been observed for a number of other, unidentified proteins in *P. putida*, *S. aureus* and *E. coli* [11]. The protein composition of total cell extracts of these species was examined by two-dimensional electrophoresis after growth under a variety of derepressive growth conditions (0.5 mM sulfur sources, including aromatic and aliphatic sulfonates and sulfates, and methionine) and compared with that for sulfate- or cysteine-grown cells. For the first two organisms, a set of 10–14 proteins was observed to be subject to regulation by the sulfur source in exactly the same way as was arylsulfatase activity. These proteins have been termed 'sulfate starvation-induced' proteins (SSI). A similar, though less extensive set of SSI proteins was also found in *E. coli* K12 (seven proteins – a correlation with arylsulfatase activity is not possible in this species) [11]. These proteins did not correlate with the known proteins of cysteine biosynthesis in *E. coli*, nor with general stress proteins whose mobilities on two-dimensional PAGE have been recorded in the *E. coli* gene-protein database [72]. The identity of the derepressed proteins in bacteria is currently under investigation. In *Neurospora crassa*, a similar regulatory system is known under the control of the CYS3 transcriptional activator [129]. The proteins derepressed in this organism during sulfur limitation are all involved in sulfur metabolism, and include arylsulfatase, cholinesulfatase and permeases for methionine, cholinesulfate and inorganic sulfate. Genetic regulation of the distribution of sulfur in the cell has also been detected in cyanobacteria. Mazel and Marlière [130] found two sets of genes encoding phycobilisomal proteins, the organism's major proteins. During sulfate deprivation, that set of genes encoding proteins with a minimum of sulfur amino acids was expressed, whereas excess sulfate in the growth medium led to production of the other set of proteins, which contained many sulfur amino acids.

What are the effectors of sulfate repression?

The best-studied pathway controlled by sulfate is that of cysteine biosynthesis in enteric bacteria, which has been the subject of recent reviews [131,132]. Sulfate, sulfide and cysteine all negatively affect enzyme synthesis in the cysteine biosynthetic pathway, though by different mechanisms. Whereas sulfide plays a direct role as an anti-inducer [133], cysteine acts in an indirect

manner [134], and the repressive effect of sulfate appears to derive from conversion to sulfide and cysteine [131]. In other sulfur-regulated systems, however, the true effectors of repression by sulfur-containing metabolites are less well known.

In *K. aerogenes*, arylsulfatase expression in a cysteine auxotroph was found to be negatively regulated by sulfate, sulfide or cysteine (1 mM) [113]. When a sulfate transport mutant was used, full repression by cysteine was observed, although now sulfate had no regulatory effect. Thus, sulfate and cysteine appear to act independently as co-repressors in this species [113]. In other organisms, regulation of arylsulfatase is simpler: in *Chlamydomonas reinhardtii* sulfate is the sole co-repressor [135], in *Neurospora crassa* cysteine plays this role [136], whereas in *Aspergillus nidulans* both cysteine and methionine are active [101].

A number of workers have carried out experiments aimed at determining the levels of sulfate or cysteine required for repression of arylsulfatase expression. Thus, in *Pseudomonas* sp. $C_{12}B$, the presence of only 10 μM cysteine or sulfate in a growth medium containing methionine (5 mM) led to a 70–80% reduction in arylsulfatase activity, and complete repression of the enzyme was observed at sulfate concentrations above 100 μM [137]. In *P. putida*, complete repression of the monooxygenolytic arylsulfonatase activity was observed with only 80 μM sulfate [91]. In several studies [104,114,137] the total sulfur concentrations supplied were between 1 and 20 mM, and hence vastly above both the growth requirements of the cells and the physiological levels normal for sulfate regulation. The results obtained must therefore be treated with some caution, since the danger of introducing inorganic sulfate as a contaminant of another sulfur source is correspondingly increased when higher concentrations are used.

Summary and Conclusions

Important phosphorus- or sulfur-containing xenobiotics degraded by bacterial cultures include the organophosphate insecticides parathion and malathion, the phosphonate herbicide glyphosate, and a variety of organosulfates and organosulfonates relevant in detergents and dyestuffs. The phosphoesterases involved in hydrolysis of parathion and malathion, arylsulfatases, alkylsulfatases and oxygenases specific for organosulfonates have been characterized, and in several instances their genes have been cloned and analysed. The enzyme responsible for the cleavage of the carbon–phosphorus bond of phosphonates such as glyphosate, however, has never been observed in vitro. Progress in the understanding of this key reaction thus has relied largely on the characterization of the genes encoding phosphonate uptake and degradation.

The degradation of phosphonate xenobiotics has largely been examined with the xenobiotic as a sole source of phosphorus for growth, since these compounds are not degraded as a source of carbon. The same applies to ionic phosphorothionates, even when the organism studied can synthesize enzymes to utilize the phosphorus-free moiety under other conditions [70]. This apparent paradox is rationalized by an understanding of the global regulatory networks in the cell. Oversimplified, enzymes responsible for scavenging phosphorus sources are expressed as part of the Pho regulon during phosphate starvation, whereas during carbon starvation an excess of phosphate is produced and the enzymes are therefore repressed. The full extent of these two global networks and their relevance for the degradation of phosphorus-containing xenobiotics has not yet been established.

The degradation of sulfur-containing xenobiotics has most often been examined with the compounds as carbon sources, and the idea that these biodegradative pathways may be independently regulated by the supply of carbon and of sulfur to the cell [11,138] is new. Preliminary evidence is only now becoming available that different enzymatic pathways may also be involved in the utilization of naturally occurring sulfonates as carbon and as sulfur sources, respectively [139]. Although there is as yet no direct genetic evidence for the existence of a global system for the control of sulfur assimilation in bacteria, observations at the protein level in a number of bacteria suggest that a regulatory sys-

tem is present which responds to sulfate limitation and controls the synthesis of proteins involved in providing sulfur to the cell. These emerging concepts, if experimentally verified, may reveal analogies between the regulation of phosphorus and sulfur metabolism.

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