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Sulfate Ester Formation and Hydrolysis: a Potentially Important Yet Often Ignored Aspect of the Sulfur Cycle of Aerobic Soils

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INTRODUCTION

The purpose of this article is to examine the potential importance of the microbial hydrolysis of sulfate esters as a means of generating inorganic sulfate (SO_4^{2-}) in aerobic, well-drained soils. In many recent articles dealing with the sulfur cycle (53, 87, 138, 150, 204-206, 212), the involvement of sulfate ester metabolism appears to have been either ignored or not examined in depth. In 1965, Dodgson and Rose (51) considered the hydrolysis of these esters to be of minor importance as a source of SO_4^{2-}. In view of data on the occurrence, formation, and hydrolysis of these esters which has accumulated since then, it is significant that these authors (53) have recently assigned to this aspect of metabolism a role of major importance for the sulfur cycle. This latter prospect will be examined here, particularly with the view of establishing a firm basis for the occurrence of ester sulfate in aerobic soils.

Previous reviews have dealt primarily with the man-made and the traditional biological sources of this anion such as the oxidation of elemental sulfur, but few attempts have been made to consider in the same article all possible sources of SO_4^{2-} for aerobic soils. Thus, in addition to sulfate ester hydrolysis, an attempt will also be made to consider these aspects as well as other less recognized sources of SO_4^{2-}, such as possible contributions made by the S-containing amino acids, the sulfonates, the sulfamates, and the sulfated-thioglycosides.

STATUS OF SULFUR IN AEROBIC SOILS

Inorganic Sulfur

The close relationship between organic carbon, total nitrogen, and the total sulfur content of soil was considered by Freney and Stevenson (92) to mean that most of the sulfur in soils throughout the world occurs in the organic rather than the inorganic state. This generalization has been substantiated by the results of soil sulfur analysis, which show (Table 1) that very little sulfur is found as S^0, S^{-2} or as SO_4^{2-}. Of 208 different soils investigated, only 5.2% (average value) of the total sulfur was present as SO_4^{2-}, and little, if any, sulfur was found in the elemental (S^0) or sulfide (S^{2-}) form (S^0 + S^{2-} represented 2.8% of total S in 14 soils). Indeed, Tabatabai and Bremner (249) were unable to detect sulfur in the latter two forms in an investigation of the sulfur content of 37 representative samples of the major soil series in

TABLE 1. *Sulfur status of soils from various geographical locations*

No. of samples	Location and depth	% SO_4^{2-}	% $S^{0,-2}$	% C-bonded S[a]	% HI-reducible S	% Ester sulfate[b]	Source
10	Eastern Australia, depth not reported	4.6	ND[c]	ND	48.3	43.7	270
1	Solonized soil	17.3	ND	ND	59.1	41.8	270
24	Eastern Australia, 0-6 inches	6.0	1.0	41.0	59.0	52.0	86, 87
3	New South Wales, Australia, 0-4 inches	1.4	ND	ND	41.4	40.0	88, 176
15	Australia, 0-10 cm	ND	ND	30.0	50.0		89
3	Australia, 0-10 cm	1.3	ND	68.7[d]	31.2[d]	31.2	90
2	New South Wales, Australia, Crookwell region, 0-10 cm						
	Virgin Podzolic soil	ND	ND	46.2	53.8		93
	Fertilized Podzolic soil	ND	ND	64.7	35.3		93
2	Same as above, different samples (?)						
	Virgin Podzolic soil	0.0	ND	56.6	43.4	43.4	91
	Fertilized Podzolic soil	0.0	ND	71.8	28.2	28.2	91
11	New South Wales, 0-10 cm	1.9	28	ND	ND		177
20	Northern Nigeria, 0-15 cm	9.8	ND	ND	52.0	42.2	32
14	England and Wales, U.K., 0-15 cm	4.4	ND	ND	ND		127
2	Calcareous soils	16.9	ND	ND	ND		127
3	Wales, U.K., 0-15 cm	0.6	ND	ND	36.2	35.6	113
3	Quebec, Canada, depth not reported	1.7	ND	ND	ND		160
7	Quebec, 0-6 inches	ND	ND	38.4	ND		161
5	Quebec, 0-6 inches	6.6	ND	35.2	59.2	52.6	158
3	British Columbia, Canada, depth not reported	2.1	ND	43.3	27.4	25.3	145, 146
1	Saskatchewan, Canada, depth not reported	0.7	ND	26.8	44.7	44.0	145, 146
64	Iowa, 0-15 cm	3.1	ND	ND	ND		248
37	Iowa, 0-15 cm	2.6	0.0	10.7	52.8	50.2	249
	"Webster" soil						
	0-15 cm	3.0	0.0	8.3	46.2	43.2	249
	30-60 cm	4.6	0.0	8.3	73.4	68.8	249
	90-120 cm	1.4	0.0	1.9	94.5	93.1	249

[a] See text for a description of and methods for determining C-bonded S (carbon-bonded sulfur) and HI-reducible S (hydriodic acid-reducible sulfur).

[b] Calculated by subtracting percentage of SO_4^{2-} from percentage of HI-reducible S. Values also corrected by subtracting percentage of S^0 and percentage of S^{2-} when determinations for S^0 and S^{2-} were reported.

[c] No determination reported.

[d] Values calculated from 1-day incubation data (Table 5; reference 90). Samples treated to remove SO_4^{2-} before reduction.

Iowa (Table 1). Although Lowe (158), and Kowalenko and Lowe (146), stressed that a third fraction may exist in some soils, soil organic sulfur is generally believed to consist of two major fractions called hydriodic acid-reducible sulfur (HI-reducible S) and carbon-bonded sulfur.

Carbon-Bonded Sulfur

Carbon-bonded sulfur is defined as soil sulfur that is reduced to S^{2-} by Raney nickel (42, 89, 161, 195). This catalyst will reduce cystine and methionine (89) but not ester sulfate (87, 89), and Freney et al. (91) found a close correlation between the concentration of Raney Ni-reducible S and the sum of the concentrations of these two amino acids in two Australian soils. It was suggested (91) that the Raney Ni-reducible fraction consisted mainly of amino acid sulfur, although this may not be true for soils in general. Thus, using the Raney Ni-reduction method (161), Lowe (159) determined the carbon-bonded sulfur content of the humic acid fraction of nine major soils in Alberta and found that only 39% of this fraction consisted of amino acid sulfur. Recent reviews dealing with the nature of the fulvic and humic acid components of soil are available (61, 117, 217, 262).

There is also evidence suggesting that some soils may contain carbon-bonded sulfur that is

not reduced to S^{2-} by Raney Ni. In an investigation dealing with 17 Australian soils, Freney and co-workers (89, 91) found that, even under optimal conditions, the amount of Raney Ni-reducible sulfur was 50% less than the theoretical quantity of carbon-bonded sulfur (calculated by subtracting the HI-reducible S from the total sulfur determined for these soils). Similar observations were reported by Lowe and DeLong (161) and by Kowalenko and Lowe (146) for Canadian soils. These results are not surprising, since Raney Ni will not reduce other compounds containing the C—S linkage such as the aliphatic sulfones or the sulfonic acids (T. H. Arkley, Ph.D. thesis, Univ. of California, Berkeley, 1961). These compounds may be present in soil (see section, Sulfonates, sulfamates, and the sulfated thioglycosides). Although our understanding of the precise nature of carbon-bonded sulfur is not clear, a consideration of this fraction is pertinent to the objectives of this article, since the sulfur-containing amino acids and the sulfonic acids can act as sources of SO_4^{2-} for these soils (see section, S-containing amino acids). In addition, the amino acids may act to regulate the synthesis of enzymes concerned with the release of SO_4^{2-} from ester sulfate (see section, Physiological Function of Microbial Sulfohydrolases). Of 99 soils that were tested (Table 1), carbon-bonded sulfur (Raney Ni-reducible S) represented, on the average, 37.4% of the total sulfur present.

Ester Sulfate

HI-reducible sulfur is more precisely defined as that fraction of organic sulfur that is reduced to H_2S by a mixture of hydriodic, formic, and hypophosphorous acids (83, 86, 234). This mixture produces H_2S only from compounds containing the C—O—S linkage (ester sulfate), C—N—S linkage (sulfamate), and from some organic sulfites such as dimethyl sulfite or diethylsulfite (86). The C—S bond is not ruptured under these conditions (83, 126). Lowe and DeLong (160) found that roughly 33% of the total sulfur of an alkaline soil extract was released as SO_4^{2-} after treatment of the dialyzed extract with 6 N HCl at 90°C for 12 h. In a more detailed study, Freney (86) quantitatively recovered the HI-reducible S present in the fulvic acid component of soil as SO_4^{2-} under similar conditions. The liberation of SO_4^{2-} after acid hydrolysis is a unique characteristic of compounds possessing either *O*-sulfate or *N*-sulfate groups (see, e.g., references 52, 212). Moreover, Freney (86) demonstrated that the sulfur present in heparin and agar (which possess these ester linkages) was quantitatively recovered as H_2S by the HI reduction method.

As further evidence for the existence of ester sulfate in soil, Freney (86) extracted a substantial quantity (45 to 81%) of the total HI-reducible S from five different soils with methanolic hydrogen chloride. This reagent is known to remove ester sulfate as methyl sulfate from the mucopolysaccharide, chondroitin sulfate (133). Although pure methyl sulfate was not isolated from the extracts, Freney (86) showed (i) that the sulfur present was not precipitated by the addition of Ba^{2+} (which normally distinguishes ester sulfate from SO_4^{2-}), (ii) that infrared spectra of the extracts exhibited absorptions characteristic of covalently bound sulfate, and (iii) that all of the sulfur present in these extracts was reducible to H_2S by the HI reduction method. According to Freney (86), these results suggested that methyl sulfate was extracted from soil treated with methanolic hydrogen chloride. It would appear that compounds bearing the C—O—S or C—N—S linkages are confined to the HI-reducible S fraction of total soil sulfur; and the general concensus (32, 86–89, 159, 160, 249) is that this fraction is largely, if not entirely, composed of N-linked or C-linked ester sulfate. Although sulfamates are not known to occur widely in nature (52), there is no evidence that such compounds are absent from soils, and Tabatabai and Bremner (249) suggested that the term "organic sulfate" be used in place of "ester sulfate" to describe the sulfur linkages present in this fraction. As in other reviews (52, 53), the term "ester sulfate" will be used in this article in reference to any compound possessing N—O—SO_3^-, N—SO_3^-, and/or C—O—SO_3^- linkages.

Since the HI reduction method will also convert S^0 and SO_4^{2-} to H_2S (83), the true percentage of ester sulfate in soil is usually obtained by subtracting the percentage of total S as S^0, S^{2-}, and SO_4^{2-} from the percentage of HI-reducible S (unless SO_4^{2-}, for example [90], was extracted before reduction). With two exceptions (86, 249), values for S^0 and S^{2-} are lacking from soil sulfur analysis (Table 1). Thus, most ester sulfate values reported here (Table 1) are not corrected for these forms of inorganic sulfur. Due to the limited concentrations of S^0 and S^{2-} in soil (Table 1), it is believed that HI-reducible S values, uncorrected for S^0 and S^{2-}, still represent a reasonable estimate of the ester sulfate content of soil. Despite these limitations, the data presented in Table 1 show that ester sulfate (corrected for S^0 and S^{2-} [86, 249] or uncorrected) represents a substantial proportion of the total sulfur in soils from various geographical locations. Of 112 different soils analyzed for SO_4^{2-} and HI-reducible S, ester sulfate represented 40.8% (average) of the total sulfur pres-

ent. The lowest value reported (25.3%) was that for a soil belonging to the black chernozemic group taken from British Columbia (145, 146), whereas Tabatabai and Bremner (249) reported values as high as 93.1% for subsoils taken from Iowa. These workers (249) further demonstrated that the percentage of total sulfur as ester sulfate increased with increases in sample depth, thus illustrating that this parameter should be considered in soil sulfur analysis in general. Available sample depth values for other studies, along with representative data from the soil profile studies of Tabatabai and Bremner (249), are included in Table 1.

Although Freney (86), in a study of several Australian soils, found that most of the HI-reducible S was associated with fulvic acid, it is now apparent (88) that this was a result of the extraction procedure. Thus, using a variety of mild extractants (176) unlikely to degrade humic acid, Freney et al. (88) found that more HI-reducible S was associated with this latter soil colloid than with fulvic acid. This result is consistent with those of Lowe (159) and Houghton and Rose (113) who found that HI-reducible S represented 39 and 51% of the total S present in humic acid isolated from Alberta and Welsh soils, respectively. In the latter study (113), the presence of sulfate ester groups in the humic acid fraction was confirmed by extraction with methanolic hydrogen chloride and by release of SO_4^{2-} after hydrolysis in 6 N HCl at 100°C. The collective results of these studies (88, 159, 176) and others (90, 91, 158) suggest that no one procedure is entirely suitable for assessing the heterogeneity of compounds comprising the HI-reducible S fraction of soil. It is likely that a significant proportion of this fraction occurs as a nonintegral part of soil colloids such as the humic and fulvic acids. Using extractants designed to remove SO_4^{2-}, Lowe (158) extracted substances from soil which he considered to be sulfated polysaccharides. Similarly, Freney and co-workers (86, 90) found that HI-reducible S could be extracted from soil using a dilute potassium phosphate solution at pH 7. Although some soils have little capacity to adsorb SO_4^{2-} (90, 248), other soils adsorb substantial quantities of this anion (14, 108, 113, 132, 158), and there is evidence that HI-reducible S may also be adsorbed to soil particles. Thus, Houghton and Rose (113) found that a wide variety of different ^{35}S-labeled sulfate esters were adsorbed to Welsh soils to the extent of 67% (average) of the total concentration of the ester that was initially added to these soils. Furthermore, procedures that do not release SO_4^{2-} from humic acid (such as heating at neutral pH [13, 234, 270] or simple grinding of soil before extraction [86]) caused a substantial increase in the SO_4^{2-} content of some soils. This observation suggests that sulfate ester linkages of differing labilities are present but are not firmly bound to soil colloids. In terms of the possible occurrence of sulfamate linkages in soil, it may be pertinent to mention that the *N*-sulfate group is more unstable than the *O*-sulfate linkage to acid hydrolysis. Thus, *N*-desulfated heparin is prepared under mild conditions that do not cause the hydrolysis of *O*-sulfate groups in this polysaccharide (52). The instability of the *N*-sulfate linkage was also demonstrated for simpler sulfamates that are excreted after the administration of arylamines to mammals (24, 196) and spiders (228). Authentic sulfate esters are known to differ in stability toward nonenzymic hydrolysis (i.e., the arylsulfates are easily hydrolyzed, whereas esters such as choline *O*-sulfate [232] and keratan sulfate [141] are reportedly resistant to autoclaving). Indeed, Segel and Johnson (224) reported that choline sulfate underwent only one-half hydrolysis after 30 min at 100°C in 1 N HCl.

Freney and co-workers (90, 91) recently demonstrated that the HI-reducible S fraction is not an inert or stable end product of sulfur metabolism in soil. Thus, when fallow soils or soils awaiting planting were incubated in the presence of ^{35}S-labeled SO_4^{2-}, ^{35}S was incorporated into both the HI-reducible S and the carbon-bonded S fractions. The HI-reducible S fraction exhibited greater specific radioactivity; using a mild extractant, Freney et al. (90) found that 75% of the ^{35}S was present in the fulvic acid component of these soils. Approximately 90% of the fulvic acid sulfur was reduced to $^{35}S^{2-}$ by hydriodic acid. Soil sterilized by autoclaving failed to incorporate ^{35}S, suggesting that microbial activity was responsible for the incorporation of the isotope (90). Plants (*Sorghum vulgare*) were found to utilize sulfur from both the HI-reducible as well as the carbon-bonded S fractions (91). Results obtained by incubating initially SO_4^{2-}-free soils, after incorporation with $^{35}SO_4^{2-}$, in the presence of *S. vulgare* suggested that immediate utilization of sulfur for growth involved the HI-reducible fraction. Thus the radioactivity present in this fraction decreased considerably during plant growth, whereas no measurable decrease occurred in the ^{35}S-labeled carbon-bonded fraction. In fact, the radioactivity in this latter fraction tended to increase, suggesting that some of the HI-reducible S was converted to carbon-bonded S during plant growth. Incubation of soils in a parallel experiment in the absence of *S. vulgare* resulted in the release of SO_4^{2-} (labeled and unlabeled) and most of this anion was de-

rived from the HI-reducible S fraction. Indeed, many of the changes observed in the carbon-bonded fraction were considered not to be significant by the authors (91). Changes in the indigenous (nonradioactive) sulfur present in these fractions were also followed during plant growth (91). The findings suggested that *S. vulgare* obtained 40 and 60% of its sulfur requirement (for a 36-week growing period) from the HI-reducible and carbon-bonded S fractions, respectively. The authors (91) stressed that this experiment did not take into account interconversion and exchange of sulfur between the two fractions. Thus, the quantity of HI-reducible S that may have been converted to carbon-bonded S is not known. It is unfortunate that this already detailed study (91) was not extended to include changes in the HI-reducible S present in the humic and fulvic acid components of these soils. These soil colloids have been considered (53) to be resistant to SO_4^{2-} release by microorganisms, but the available evidence for this is still not convincing. In the only reported study of the degradability of humic acid sulfur, Houghton and Rose (113) found that the sulfate ester groups present in this colloid were resistant to hydrolysis by extracts possessing alkylsulfatase, arylsulfatase, and glycosulfatase activities. It is well established that depolymerization occurs before the desulfation of other sulfated macromolecules (52, 53) and, as the authors (113) pointed out, the desulfation of humic acid may result from a sequential attack by depolymerizing and desulfating enzymes.

ORIGINS OF SOIL ESTER SULFATE

Mammalian Sources

The presence of high concentrations of ester sulfate in soil is not surprising in view of numerous reports dealing with the natural occurrence of these esters. Mammalian connective tissue consists of keratan sulfate (keratosulfate), dermatan sulfate, and chondroitin sulfate as well as heparin and heparan sulfate. The last two polysaccharides possess the C—O—S and the C—N—S (sulfamate) linkage and represent the only known naturally occurring substances with the latter sulfur linkage (52). Recently, Rahemtulla and Lovtrup (208, 209) demonstrated the presence of chondroitin sulfate in many invertebrates that inhabit soils. Collectively, these polysaccharides are released into soil from decaying animal matter. A number of sulfate esters are returned to soil in animal exreta (arylsulfates [1, 34, 35, 38, 52, 57, 110], sulfate esters of steroids [97, 99, 101, 175, 215, 260], the amino acid *O*-sulfates [112, 125, 253] and ascorbic acid 2-*O*-sulfate [10, 183]). To emphasize the magnitude of this latter contribution, Dodgson and Rose (53) stated that "a rough estimate suggests that in terms of human excreta alone almost 50 tons of sulfur are daily returned to the sulfur cycle in the form of sulfate esters." Although the above-mentioned esters probably represent the major contribution of the mammals to the organic sulfate content of soil, other esters of animal origin may be returned to soil less frequently. These include the sulfate esters of glycoproteins (59) and of bile alcohols (23, 231), lactose 6-*O*-sulfate and neuramin lactose 6-*O*-sulfate of mammary glands (12, 213), heparin from mast cells (53), the mammalian sulfated glycolipids (80, 124, 242), the polyhexose sulfate esters of primitive animals (55, 116, 122, 123, 137), uridine 5'-diphosphate-*N*-acetyl-D-galactosaminesulfate, and isopropyl sulfate from the hen's oviduct (240) and egg (274), respectively.

Microbial and Plant Sources

To these animal sources of ester sulfate can be added a growing list of esters that are synthesized by other forms of life. Taylor and Novelli (Bacteriol. Proc., p. 190, 1961) reported that an unidentified bacterial isolate from soil was capable of synthesizing an extracellular polysaccharide possessing ester sulfate groups. Sulfate esters of short-carbon-chain monocarboxylic acids similar to those formed and released into the medium by *Pseudomonas fluorescens* (67–69) were also detected in soils incubated with ^{35}S-labeled D-glucose 6-*O*-sulfate (C. Houghton, personal communication). These acids may also occur in soils as a consequence of the mammalian (44, 194) and microbial (56) degradation of the primary alkylsulfate detergent, sodium dodecyl sulfate. Both primary and secondary alkylsulfate esters are employed as components of commercial surface active agents (5, 21) and, in at least one case, an alkylsulfate ester was used as a herbicide (266). Other alkylsulfate esters were detected in a variety of microorganisms (100, 171, 172), and the possibility that naturally occurring alkylsulfates are widely distributed is strengthened by the observations that mammals (231, 263) and lower vertebrates (222) are capable of sulfating a wide variety of alcohols.

Burns and Wynn (27) recently demonstrated that extracts of *Aspergillus oryzae* can synthesize tyrosine *O*-sulfate as well as a number of other arylsulfate esters. Phenol sulfotransferase activity was thought heretofore to be restricted to mammals (98, 170, 192, 212, 223), but it appears now that the fungi and possibly the

bacteria as a group may also possess this type of activity. In this regard, Burns and Wynn (27) stressed that the presence of phenol sulfotransferase in *A. oryzae* suggests that sulfate esters of phenols may be of more widespread occurrence in microorganisms than has hitherto been thought. The point that should be borne in mind when assessing the natural occurrence of any sulfate ester is that the demonstration of sulfotransferase activity depends, in may cases, upon the fortuitous choice of a suitable sulfate donor and alcohol acceptor. We may therefore be underestimating the number and variety of organisms contributing to the production of these esters. The additional occurrence of compounds possessing the N—O—SO_3^- linkage (sulfated thioglycosides) in most cruciferous plants (142, 264) and of bacterial lipids bearing sulfate ester linkages (95, 96, 102, 135, 136, 147) suggests that nonmammalian sources of ester sulfate may be prevalent in soil. The actual magnitude of this contribution will not be appreciated until investigations are conducted to assess the ability of various microorganisms to synthesize these esters. In many cases, the observation of the biological occurrence of a sulfate ester is accidental, arising as an interesting sideline from a sometimes totally unrelated study. My observation (64) that bacteria can synthesize the *O*-sulfate ester of choline (see below) represents a good case in point.

Generation of Choline Sulfate

Choline *O*-sulfate (choline sulfate) is returned to soil from a variety of sources. This ester was found in high concentration as a constituent of lichens (60, 107, 154, 230), algae (121, 155, 257), plants (190, 258), and numerous fungi (11, 15, 30, 104). Since choline sulfate-producing fungi are able to transport the ester even under conditions that permit intracellular synthesis, this ester was considered as an important source of sulfur for microbial growth (15) and, by virtue of its resistance to nonenzymic hydrolysis (64, 224, 232, 250), as an important nonacid storage form of soil SO_4^{2-}. Sulfate esters can occur in the free acid form; but choline sulfate is an internally compensated salt. As such, its presence in soil does not alter pH.

In agreement with the results of Ballio et al. (11), choline sulfate was found to be synthesized by most of the higher fungi but was absent in members of the orders *Mucorales* and *Endomycetales* when these fungi were grown in enriched media supplemented with sodium sulfate (104). Even when grown under conditions in which the sulfate for choline sulfate must be derived endogenously from taurine, the ester can accumulate in the mycelia of *Aspergillus nidulans* to a concentration of 0.6% of the dry weight (233). Similar results (0.2 to 0.3% of the dry weight) were reported for *A. sydowi* (273) and *Penicillium chrysogenum* (39, 238). In contrast with various *Pseudomonas* species that form this ester (see below), choline sulfate was not detected in culture filtrates of the choline sulfate-producing fungi (39, 104, 238, 273). The formation of the ester in fungi required the initial formation of 3'-phosphoadenylyl sulfate (131, 232), with the subsequent transfer of SO_4^{2-} to choline catalyzed by choline sulfokinase (232). Orsi and Spencer (193) purified the choline sulfokinase from *A. nidulans* and demonstrated that this enzyme did not require Mg^{2+}. They suggested that this cation was required for 3'-phosphoadenylyl sulfate formation rather than for choline sulfate formation per se. The activity of choline sulfokinase of *A. nidulans* (193) was not inhibited by L-cysteine. However, factors regulating the synthesis of this and other enzymes of the choline sulfate pathway in fungi remain to be determined.

Choline sulfate is also present in conidiospores of several species of *Aspergillus*, accounting for as much as 1.5% of the dry weight and 40% of the total sulfur present. Spores containing similar quantities of choline sulfate were formed when either an enriched but SO_4^{2-}-unsupplemented medium or a synthetic medium containing Na_2SO_4 was employed (250). In contrast, the formation of this ester by mycelia and conidia of *Neurospora crassa* is related to the SO_4^{2-} content of the environment. Thus, a 98% increase in the choline sulfate content of both developmental stages was observed after growth on 2.0 mM Na_2SO_4, as opposed to growth in the presence of 0.1 mM Na_2SO_4 (173). No similar increase occurred when conidia were formed in media containing increasing concentrations of L-methionine. Ascospores of *N. crassa* possessed the highest levels of choline sulfate (80% of the total soluble S), but the formation of the ester by this developmental stage was independent of the external SO_4^{2-} content of medium before and for periods up to 40 h after germination (173). When various choline sulfate-producing fungi were grown under conditions that permit choline sulfate formation (0.05% [wt/vol] $MgSO_4$) and then transferred to a sulfur-deficient medium, the fungi continued to grow, whereas, in a parallel experiment, fungi incapable of forming the ester failed to grow further in the sulfur-deficient medium (232). Similarly, ^{35}S-labeled conidiospores of *A. niger* were observed to undergo complete germination in a sulfur-free

medium (252, 276). Under these conditions, about 50% of the total radioactivity present in the choline sulfate of the spores was recovered in various sulfur-containing amino acids after germination (252). These findings led Spencer and Harada (232) and Takebe (250) to conclude independently that choline sulfate acts as a reserve source of sulfur for fungal growth and conidiospore germination, respectively.

Choline sulfate may also occur in soils as a result of bacterial synthesis. Of eight randomly selected *Pseudomonas* species, all formed the ester when cultured on growth-limiting concentrations of SO_4^{2-} (64). Unlike fungi, which retain most of the choline sulfate that is synthesized, a large proportion of the ester was released by these bacteria into the culture medium. Factors regulating the formation of choline sulfate were investigated further (64) using a *Pseudomonas* species isolated from soil (197, 198), and with this isolate (designated as *Pseudomonas* $C_{12}B$ [198]), the ester was found in the culture medium at all stages of the culture cycle. Maximum quantities were discerned in stationary-phase culture supernatants (64). Adenosine 5'-triphosphate and Mg^{2+} were required for the formation of the ester by cell extracts. In this respect, the choline sulfate synthesizing system in *Pseudomonas* $C_{12}B$ (64) is similar to that present in various fungi (130, 131, 232).

The exact mechanism for the formation of the ester by *Pseudomonas* $C_{12}B$ is presently undefined. It is known (64) that this system is not repressed by the presence during growth of SO_4^{2-} or L-methionine but is inhibited in vitro by L-cysteine. These results were interpreted (64) to mean that, at growth-limiting concentrations of SO_4^{2-}, the endogenous concentration of cysteine was not sufficient to inhibit choline sulfate formation. Choline sulfate has also been detected as an intracellular component of *Lactobacillus plantarum* when this bacterium was grown in an enriched medium in the absence of added sulfur-containing compounds (120).

MINERALIZATION OF ESTER SULFATE

The reentrance into the sulfur cycle of SO_4^{2-} immobilized in organic sulfate esters is dependent upon the ability of microorganisms, plants, and mammals to produce enzymes (sulfohydrolases) that hydrolyze these esters. Bacteria and fungi appear to be the major source of these enzymes in soil. However, plant roots are known to hydrolyze choline sulfate (188), and the possibility exists that mammalian urine may contain such enzymes. It is known that arylsulfatase is present in human urine in quantities that permit the purification of the enzyme from this source (26, 239). The almost ubiquitous occurrence of this enzyme in the organs of mammals (51, 52, 212) suggests the likelihood of its eventual discovery in the urine of other mammals.

It has been known for some time that soils contain a number of different classes of enzymes that originate from, but exist outside of, living tissue (for a review, see [227]). Recently, it was demonstrated that sulfohydrolases, present in soils, are capable of desulfating esters of many different types. Cooper (32) found that wetting Nigerian soils caused the release of SO_4^{2-}, and that SO_4^{2-} liberation was associated with a corresponding decrease in the size of the HI-reducible S fraction of these soils. Inorganic sulfate release was inhibited completely only in the presence of compounds that both suppress microbial growth and inhibit the activity of extracellular enzymes. Twenty different soils were found to contain arylsulfatase (arylsulfate sulfohydrolase EC 3.1.6.1) activity, and in all cases the activity was positively correlated with the HI-reducible S fraction of each soil (32).

Although arylsulfatase plays a major role in mineralizing ester sulfate for the sulfur cycle in Nigerian soils, there are suggestions that this particular sulfohydrolase may not be involved to the same extent in soils from other parts of the world. Thus, Tabatabai and Bremner (245–247) assayed 21 different Iowa soils and found that all samples possessed appreciable arylsulfatase activity. Soils sterilized by gamma irradiation still possessed the enzyme, but at reduced levels, suggesting that a large proportion of the activity was due to arylsulfatase present in or released by nonviable microorganisms (245). This result implies a high degree of stability for the enzyme, and Tabatabai and Bremner (246) reported only an 18% decrease in arylsulfatase activity of field moist soils after storage at 22 to 24°C for 3 months. This enzyme also appears to function well under adverse conditions of temperature. Optimal activity for arylsulfatase in six different soils was detected at an incubation temperature of roughly 67°C (245). Stability studies with the purified enzyme from microbial sources complement these findings (see section, Sulfohydrolase stability in vivo and in vitro). Factors other than arylsulfatase content appear to be involved in mediating SO_4^{2-} release from Iowa soils. Although arylsulfatase activity was significantly correlated with the organic carbon content of each soil examined (246), this activity was not significantly correlated with the total amount of

SO_4^{2-} released after incubation of these soils in the absence of added substrate. For example, only 0.6% of the total S was mineralized in the soil with the highest initial level of arylsulfatase after aerobic incubation at 30°C for 10 weeks. However, 3.0% of the total S was converted to SO_4^{2-} in the soil with the lowest initial level of the enzyme (248). Unfortunately, the HI-reducible S content of these particular samples was not reported (246, 248). Kowalenko and Lowe (146) found that that a Prest soil from British Columbia (145) possessed the highest initial level of arylsulfatase of four soils examined and demonstrated that this same soil also released the greatest amount of SO_4^{2-} during a 14-week incubation period in the absence of added substrate. Maximum SO_4^{2-} release occurred during the first 2 weeks of incubation, after which a plateau was reached. Moreover, it was also shown that the arylsulfatase activity was correlated with SO_4^{2-} release over the entire incubation period. However, the arylsulfatase activity of this soil decreased sharply over the first 4 weeks, and the authors (146) concluded that the presence of this enzyme was not a major factor controlling the release of SO_4^{2-} in this soil. Similar results were obtained with the other three soils examined, but supporting data were not presented (146). As will become apparent (see section, Physiological Function of Microbial Sulfohydrolases), microbial arylsulfatase synthesis is subject to end product regulation by SO_4^{2-}. If microbial activity is responsible for SO_4^{2-} release under these conditions, then a decrease in the intracellular as well as the extracellular levels of the enzyme would be expected once maximum SO_4^{2-} release was achieved.

Arylsulfates are not the only sulfate esters present in soils (see section, Origins of Soil Ester Sulfate), and it follows from this that, irrespective of the involvement of arylsulfatase, other sulfohydrolases may also be responsible for sulfur mineralization in soil. Such a possibility is currently being investigated in the laboratories of F. A. Rose, and this work to date shows that Welsh soils (Table 1 and references 53 and 113) possess enzymes capable of hydrolyzing ^{35}S-labeled choline sulfate, dodecyl sulfate, glucose 6-*O*-sulfate, and tyrosine *O*-sulfate, as well as arylsulfate esters such as 2-hydroxy-5-nitrophenyl sulfate and phenyl sulfate.

It can also be demonstrated that for every sulfate ester that could be found in soil, at least one soil microorganism has been isolated that releases SO_4^{2-} from that ester. By far the greatest effort has been directed toward a study of arylsulfatase, and there are numerous detailed reports on the occurrence of this enzyme in bacteria (41, 47, 74, 78, 81, 106), fungi (8, 17, 28, 105, 157, 181, 214, 221), and algae (152). Other enzymes of microbial origin have been reported to desulfate mono-, di-, and tetrasaccharide sulfate esters (149, 156, 211, 225, 268, 275), carbohydrate-related sulfate esters (68, 69), polysaccharide sulfate esters (46, 140, 184), amino acid sulfate esters (76, 103, 241, 261), alkylsulfate esters (56, 70, 114, 199, 200, 201, 202, 241), and choline sulfate (79, 103, 118, 120, 162, 224, 241, 251).

PHYSIOLOGICAL FUNCTION OF MICROBIAL SULFOHYDROLASES

To asses the involvement of microorganisms in the conversion of ester sulfate to SO_4^{2-}, it is necessary to know: (i) the physiological (environmental) factors that regulate the synthesis of the enzymes concerned with SO_4^{2-} release, (ii) the location of these enzymes within the cells that produce them, and (iii) the stability characteristics of the enzymes when they exist outside of the cell.

Regulation of Sulfohydrolase Synthesis and Activity

With some exceptions (70, 73, 275), the sulfohydrolases (sulfatases) are not constitutive in bacteria or fungi. The synthesis of these enzymes is controlled by either the sulfur or carbon content of the environment. *Pseudomonas* $C_{12}B$ (a soil isolate) synthesizes two substrate-inducible primary alkylsulfatases, P1 and P2 (65, 71, 76, 271), and a secondary alkylsulfatase (S3) whose formation is induced by sulfate esters of secondary alcohols in the presence of the corresponding parent alcohol (48). The induction of none of these enzymes is sulfate ester specific, and the synthesis of the P2 form of primary alkylsulfatase, for example, is induced by secondary as well as primary alkylsulfates (48).

Knowledge of factors controlling sulfatase induction is necessary for an assessment of the relative importance of these enzymes in sulfate ester mineralization. For example, sulfatase induction that is regulated by the carbon or by the sulfur status of soil would be expected to have a greater role in the microbial mineralization process than would an enzyme whose synthesis was inhibited in the presence of both carbon- and sulfur-containing compounds. An example of this latter circumstance is the formation of choline sulfatase in *Pseudomonas* 5-A (a sewage isolate). The induction of this enzyme by choline sulfate is inhibited by SO_4^{2-}, cysteine, methionine, and choline chloride (79).

Microorganisms having sulfohydrolase enzymes regulated in this way will synthesize only enough enzyme to satisfy their growth requirement. A less rigidly controlled system is the induction of both primary and secondary alkylsulfatases in *Pseudomonas* $C_{12}B$. The formation of these enzymes was unaffected by the presence or absence of SO_4^{2-} or the S-containing amino acids but was inhibited by some alcohols and Krebs cycle intermediates (77). Fitzgerald and Payne (77) suggested that the main function of these enzymes was to obtain carbon and energy for the growth of *Pseudomonas* $C_{12}B$. This possibility may also apply to other alkylsulfatase-producing bacteria of soil origin. Thus, the synthesis of secondary alkylsulfatase by *Comamonas terrigena* is unaffected by the sulfur status of the culture and is confined exclusively to the stationary phase of the culture cycle (70). These results suggest that a depletion of carbon triggers the formation of the enzyme. Similarly, primary alkylsulfatase induction in a recently isolated soil bacterium is subject to inhibition by glucose, catabolites of glucose, and by adenosine 5'-triphosphate (unpublished data, these laboratories).

The same type of sulfatase may be regulated differently depending upon whether it is synthesized by bacteria or fungi. Thus choline sulfatase is substrate inducible in bacteria (79, 103, 162, 251), but its synthesis in *A. nidulans* (219, 233) and *N. crassa* (168, 174, 178) is regulated by a sulfur-mediated derepression mechanism. Although the enzyme was synthesized only when these fungi (mycelial stage) were cultivated in sulfur-deficient media, this was not true for the choline sulfatase of *Penicillium chrysogenum*. Lucas and co-workers (162) found that the formation of this enzyme by mycelia was repressed to only 20% of the maximum level by excess SO_4^{2-} or methionine. The work of McGuire and Marzluf (174) demonstrates that factors regulating the synthesis of choline sulfatase may vary depending upon the fungal developmental stage. Although choline sulfatase formation by *N. crassa* during the mycelial stage was repressed by 2 mM methionine or SO_4^{2-}, the same concentration of SO_4^{2-} was ineffective as a repressor of the synthesis of this enzyme by *N. crassa* conidia. Choline sulfatase formation by conidia was regulated only by the methionine content of the medium with full repression and derepression taking place at 5 and 0.25 mM, respectively (174).

The sulfohydrolases that are required for the degradation of heparin (45) and keratan sulfate (140, 184) are induced respectively by these mucopolysaccharides, but reports on factors regulating the induction process are unavailable.

Arylsulfatase formation by many bacteria (4, 78, 103, 106, 180, 210) and fungi (53, 105, 119, 178, 181) is repressed by SO_4^{2-} and, in most cases, by other components of the cysteine biosynthetic pathway. The actual co-repressor(s) is not known with certainty for any one system, and some evidence suggests that different effectors may be involved. Thus, work with cysteine auxotrophs suggested that SO_4^{2-} was most directly involved in repressing arylsulfatase formation by *Aerobacter* (*Enterobacter*) *aerogenes* (210). However, cysteine was much more effective than SO_4^{2-} as an effector regulating the synthesis of this enzyme in *Pseudomonas* $C_{12}B$ (78) and, based upon work with a number of mutants of *Klebsiella aerogenes*, Adachi et al. (4) suggested that both SO_4^{2-} and cysteine acted independently to repress arylsulfatase formation in this particular bacterium. Harada and Spencer (105) suggested that SO_4^{2-} was the co-repressor of arylsulfatase synthesis by a number of fungi, but Metzenberg and Parson (178) suggested that S^{2-} was most directly involved in repressing the synthesis of this enzyme by *N. crassa*.

Derepression of arylsulfatase formation occurs when various bacteria are grown on methionine as the sole source of sulfur (4, 78, 106, 180) and, for the synthesis of this enzyme by *A. aerogenes*, Rammler et al. (210) interpreted this result to mean that methionine is not converted directly (or rapidly) to cysteine in this bacterium. In these studies, the various bacteria responded to increasing concentrations of methionine by synthesizing increasing levels of the enzyme. Methionine does not exert a similar effect on arylsulfatase formation by fungi. This amino acid acted as a repressor at high (5 mM) concentrations (105, 168, 174, 178), and derepression occurred when *N. crassa* was grown on low (0.25 mM) concentrations (168, 174). Metzenberg and Parson (178) suggested that methionine acted independently of S^{2-} as an additional co-repressor of arylsulfatase formation in this latter fungus. In light of results obtained by Benko et al. (16), it is not surprising that methionine and components of the cysteine pathway might act independently in this way. Thus, in a study of the transport of ^{35}S-labeled methionine by various fungi, these workers (16) found that most of the isotope was associated with cystathionine and little, if any, ^{35}S-labeled cysteine or cystine was detected in mycelial extracts. The regulation of arylsulfatase synthesis is equally complex in motile and nonmotile strains of *A. aerogenes*. The forma-

tion of the enzyme in these bacteria is controlled not only by the sulfur status of the environment (4, 106, 210) but also by glucose-mediated catabolite repression (3) and derepression by tyramine (2).

The oceans are rich in SO_4^{2-} (see, e.g., reference 138), and there are indications of the existence in marine environments of arylsulfatase-forming systems of microbial origin that are refractive to repression by SO_4^{2-} or cysteine. Thus, marine sediments possess arylsulfatase activity (31), and Dodgson et al. (50) isolated a strain of *Alcaligenes metalcaligenes* from this source which, when grown in nutrient broth, was capable of synthesizing arylsulfatase in quantities suitable for its isolation (54). This enriched medium generally contains enough sulfur to repress the synthesis of arylsulfatase by other bacteria. The finding that the inclusion of tyramine in the medium caused a substantial increase in the arylsulfatase activity of undialyzed extracts of *A. nidulans* was interpreted as an effect on arylsulfatase synthesis (119). This possibility should be reinvestigated, since Burns and Wynn (27) recently found that tyramine caused an activation per se of two of the arylsulfatase isozymes in *A. oryzae*. This latter study also stresses the fact that the inclusion of hydroxyl-containing compounds in sulfohydrolase assay media should be avoided unless it is known that the enzyme does not possess a sulfotransferase function for that compound. In *A. oryzae*, "arylsulfatase II" exhibited sulfotransferase activity not only for tyramine (27) but also for a number of other phenols (see also section, Microbial and plant sources), thus accounting for the apparent activation of the enzyme with respect to its sulfohydrolase activity.

Arylsulfatase formation may also be derepressed when bacteria (40, 78, 210), fungi (9, 43, 119, 168, 174, 181, 220), and algae (218) are cultivated in sulfur-deficient media. Consequently, it has been suggested (53, 210) that the function of this enzyme is the provision of SO_4^{2-} for microbial growth in SO_4^{2-}-deficient environments containing arylsulfate esters. These results may explain why SO_4^{2-}-deficient soils (see section, Mineralization of Ester Sulfate and Table 1) possess appreciable levels of the enzyme. However, small differences in arylsulfatase activity among the soils assayed (32, 146, 246, 248) were not related to either the total sulfur content or the SO_4^{2-} content of these samples. Since the soils contained high levels of carbon-bonded S (Table 1), it is likely that cysteine and methionine may also be present. These amino acids may act to regulate sulfate ester mineralization in soil mediated by microorganisms that are able to synthesize either arylsulfatase or choline sulfatase. The concentrations of cysteine and/or methionine required for complete repression or derepression of arylsulfatase formation by a bacterial culture can be as low as 10^{-2} mM (for repression by cysteine) or 10^{-4} mM (for derepression by methionine) (78). Unfortunately, the ability of soils to hydrolyze choline sulfate has been reported only once (113), and data correlating soil arylsulfatase activity with the cysteine and/or methionine content of the same soils is unavailable. In addition, it is not known to what extent these amino acids occur free of peptide bond linkage to other amino acids in the soils where they have been detected (91, 93).

Localization of Sulfohydrolase Activity

A knowledge of the location of the sulfohydrolases in microorganisms is essential to evaluate the contribution made by viable cells to sulfate ester mineralization in soil. If a particular sulfohydrolase is not found on the cell periphery, then its action on a sulfate ester will more likely yield SO_4^{2-} for microbial growth rather than plant growth. For example, primary alkylsulfatase is cell wall associated in *Pseudomonas* $C_{12}B$ (73), and high concentrations of the enzyme (73) and SO_4^{2-} (71) were found in the culture medium when this soil isolate was grown in the presence of a primary alkylsulfate ester. Similar considerations apply to the secondary alkylsulfatases of this isolate (72, 73) and to the choline sulfatase present in another *Pseudomonas* isolate (79). Inorganic SO_4^{2-} accumulated in the medium during the growth of this latter bacterium on choline sulfate (79). The sulfohydrolases involved with heparin (45) and keratan sulfate degradation (141) may also be cell wall associated, since roughly 50% of the total activity after growth was found in the culture medium. Unlike many other enzymes (see e.g., references 163, 255, 256), the alkylsulfatases are resistant to denaturation in the presence of sodium dodecyl sulfate (K. S. Dodgson, this laboratory, unpublished data; 53). These enzymes share this unique characteristic with alkaline phosphatase (169), and there are good theoretical reasons for expecting that enzymes that deal with protein-dissociating agents such as sodium dodecyl sulfate (267) would be located on the cell periphery as are other hydrolases (see, e.g., references 185, 186, 187, 269). It may be unwise, however, to generalize at this stage, especially since the secondary alkylsulfatases of *Comamonas terrigena* were not released by osmotic

shock or during spheroplast formation (70). Recent unpublished data obtained from membrane vesicles by G. Matcham in this laboratory suggest that these enzymes are associated with the cytoplasmic membrane (inner membrane) in this bacterium.

Although concrete visual evidence is lacking, the fact that arylsulfatase can be assayed using intact cells or mycelial pellets suggests that this sulfohydrolase also occupies an exocytoplasmic location within the bacterial cell (4, 179, 210, 241), the algal cell (152), the fungal mycelium (43, 157, 181), and the fungal conidium (220). Rammler et al. (210) used intact cell suspensions to measure arylsulfatase activity in *A. aerogenes* and reported a complete recovery of activity in the supernatant after cell rupture and centrifugation. With the exception of the temperature optimum, Lien and Schreiner (152) found that arylsulfatase, present in whole cells of *Chlamydomonas reinhardti*, was similar in a number of properties to the same enzyme purified to the single-protein stage from this source. No increase in activity accompanied cell rupture, and the authors (152) suggested that the enzyme was attached to the cell surface of this alga. Preliminary results of an investigation of the cytochemical location of the enzyme (referred to in reference 152) supported this conclusion, but these results have not, as yet, been published. Rammler et al. (210) concluded that arylsulfatase was also located on the surface of *A. aerogenes*, and a similarity in properties of the enzyme present in intacts cells as opposed to cell extracts of *Proteus rettgeri* was also noted. In these latter studies, the same substrate-dependent activation of arylsulfatase by PO_4^{3-} was observed with intact cells (179) and with partially purified cell extracts (75). Results of work by Metzenberg's group indicate that the location of this enzyme in *N. crassa* is dependent upon which developmental stage is examined. Thus, in young mycelia arylsulfatase is associated with particles resembling the lysosomes of mammalian cells (221), but in conidia the enzyme was found to be surface associated as well as intracellular (220). Approximately 70% of the arylsulfatase present in *Pseudomonas* $C_{12}B$ (see reference 78) was released either after osmotic shock or during spheroplast formation. This suggests that the enzyme is cell wall associated in this soil isolate (unpublished data, this laboratory).

Data from studies of the transport of choline sulfate by fungi (15, 33, 166, 233) suggest that choline sulfatase is not cell surface associated in these microorganisms. Bellenger et al. (15) found that a mutant of *Penicillium notatum*, deficient in SO_4^{2-} activation, accumulated choline sulfate to an intracellular concentration of 0.075 M after suspension for 3 h in a medium containing 5 mM ester. The intracellular concentration of SO_4^{2-} (from the subsequent hydrolysis of the ester) was 0.035 M, and no SO_4^{2-} was detected in the assay medium (15). Choline sulfate was transported unchanged by mycelia when short (2-min) incubations were employed. The transport of this ester by *P. notatum* as well as by a number of other fungi (15) is due to a highly specific permease whose synthesis in *P. notatum* and *P. chysogenum* is regulated by a sulfur-mediated repression-derepression type of control similar to that regulating the synthesis of some sulfohydrolases (see section, Regulation of Sulfohydrolase Synthesis and Activity). In a similar study, Marzluf (166) used a mutant of *N. crassa*, deficient in SO_4^{2-} transport, to confirm that the intact ester (and not its hydrolysis products) was taken up by mycelia. Inorganic sulfate was not detected in the assay medium when this mutant was incubated with choline sulfate for 2 h. It was concluded (166) that extracellular hydrolysis did not take place. Using a mutant, incapable of activating SO_4^{2-}, Marzluf found that 70% of the transported choline sulfate was hydrolyzed by *N. crassa* intracellularly during this time interval. *N. crassa* also has a specific choline sulfate permease, and the synthesis of this transport protein is also regulated by the sulfur status of the culture, being repressed by methionine or SO_4^{2-} (166). These effectors did not inhibit transport activity (166, 167). It is important in terms of assessing the contribution to the SO_4^{2-} content of soils made by viable fungi to point out that liberated SO_4^{2-} was observed to be retained by mycelia against a concentration gradient in both investigations (15, 166). These results disagree with those obtained with bacteria in which excess SO_4^{2-} originating from the hydrolysis of primary alkylsulfate (71) or choline sulfate (79) was found in the culture medium. Although choline sulfatase may have a surface location in this latter isolate, Nissen (188) reported that a wide variety of other soil bacteria were capable of transporting choline sulfate. The ability or inability of these bacteria to hydrolyze the ester was not reported.

Potassium D-glucose 6-*O*-sulfate (glucose sulfate), an ester that is analogous to those comprising some sulfated polysaccharides (49), is utilized by *N. crassa* via a mechanism that also involves the transport of the ester followed by its hydrolysis within the mycelium (211). A mutant of this fungus that is deficient in SO_4^{2-} transport was nevertheless capable of trans-

porting glucose sulfate and of growing on this ester as a sulfur source (211). The observation that SO_4^{2-} could not be detected in the transport assay medium even after a 6-h incubation was interpreted to mean that the SO_4^{2-} required for growth was obtained by intracellular hydrolysis. Although SO_4^{2-} was found to inhibit the transport of glucose sulfate, the uptake system for this ester is distinct from those that act to transport SO_4^{2-} (165), choline sulfate (166), or glucose (216). The glucose sulfate system is subject to a repression-derepression type of control mediated primarily by methionine (211). It is fortunate that tris(hydroxylmethyl)aminomethane was not used as a buffer component for these transport studies (211), since it was recently demonstrated that this primary amine (≧25 mM) can catalyze the isomerization of glucose sulfate as well as the partial hydrolysis of the resulting fructose sulfate (66). In work that established for the first time the existence of a glycosulfatase in fungi, Lloyd and co-workers (149, 156) found that *Trichoderma viride* released SO_4^{2-} into the culture medium when grown on the 6-*O*-sulfate esters of either glucose or galactose. Since no attempt was made to localize the sulfohydrolase in this study, it is not known whether these results indicate a surface location for the enzyme or whether this fungus differs from *N. crassa* (211) and various *Penicillium* spp. (15) in being unable to retain the SO_4^{2-} that was generated by intracellular hydrolysis.

Sulfohydrolase Stability In Vivo and In Vitro

Obviously, a soil microorganism having a sulfohydrolase that lacks pH and/or thermostability will make little contribution to the sulfur cycle in terms of sulfate ester mineralization. Although published data are unavailable on the stability of these enzymes in a crude state, it has been noted in this laboratory and elsewhere that the sulfohydrolases as a class appear to be very stable to elevated temperature when present in crude cell extracts. Presumably in this state, the enzyme is protected from denaturation by the presence of extraneous proteins and polysaccharides. Soils are known to contain these substances in addition to a highly complex mixture of other organic compounds of unknown origin (see, e.g., references 25, 63) and the protective environment found in soil may be similar to that existing in a crude cell extract. Previous reference has been made to the stability of arylsulfatase in soil samples (see section, Mineralization of Soil Ester Sulfate).

Results of stability studies on arylsulfatase, purified to the single-protein stage, attest to the inherent durability of these proteins. Thus, no appreciable loss in activity was noted when the enzyme (purified from *Chlamydomonas reinhardti*) was incubated for 16 min at a temperature as high as 60°C. The optimum temperature for activity of arylsulfatase in intact cells was also 60°C (152). The α and β isoenzymes of arylsulfatase from *Pseudomonas aeruginosa* were stable to the same temperature for 5 min (41). Delisle and Milazzo (41) also observed these enzymes to be equally stable to pH over the range 6.5 to 10.0 for periods of storage at 37.5°C up to 24 h. The enzymes also exhibited appreciable activity over this pH range. Similar results were obtained with a partially purified source of the enzyme from *A. aerogenes*. In this study, Fowler and Rammler (81) found, in addition, that this enzyme was maximally active over a wide temperature range extending from 28 to 60°C. With the exception of work by Payne et al. (201), stability studies of other purified sulfohydrolases of microbial origin have not been reported. In this study, it was demonstrated that primary alkylsulfatase, present in $(NH_4)_2SO_4$-fractionated extracts of *Pseudomonas* $C_{12}B$, hydrolyzed sodium dodecyl sulfate best at 70°C. This enzyme was stable in the presence of its substrate for 1 h at 60°C (201). Primary alkylsulfatase was active over a pH range of 5 to 9, but its stability over this pH range was not reported. It may be anticipated from observations made with crude cell extracts that similar studies with other purified sulfohydrolases will reveal these enzymes to be inherently stable as well.

SOURCES OF INORGANIC SULFATE FOR AEROBIC SOILS

Elemental and Sulfide Sulfur

Although the oxidation of reduced forms of sulfur (S^0 and S^{2-}) represents a major mechanism for the generation of SO_4^{2-} in anaerobic environments (see, e.g., references 62, 129, 150, 205, 206), there is evidence suggesting that this is not necessarily the case for well-drained terrestrial soil. Thus, although many bacterial photolithotrophs are capable of these oxidations in aquatic environments, soil is not a suitable medium for their survival. Light and anaerobiosis cannot occur together in this kind of environment. The aerobic oxidation of S^0 is carried out principally by three species of the genus *Thiobacillus*, and the ability to oxidize S^{2-} appears to be even further restricted, being confined to a single bacterial species, *Thiobacillus thioparus* (58). Indeed, over 50% of the 329 Aus-

tralian surface soils investigated by Swaby and co-workers oxidized added S^0 either very slowly or not at all (243, 244, 265). The inability to oxidize this form of sulfur was correlated with the absence of the thiobacilli from these soils (244).

Sulfate Ester Hydrolysis

The occurrence of sulfate esters in aerobic soils and the additional presence of microorganisms capable of liberating SO_4^{2-} from these esters suggest that sulfate ester hydrolysis does represent a source of this anion for the sulfur cycle. This possibility is further substantiated by the stability characteristics of the sulfohydrolases that catalyze this reaction and by the cell surface location of many of these enzymes. However, due to the limited data available, it is not possible at present to evaluate the extent of this contribution to the sulfur cycle. Certainly the hydrolysis of these esters may represent a major means of generating SO_4^{2-} in Australian soils that lack the thiobacilli (244), but contain high concentrations of ester sulfate (Table 1). Detailed studies on sulfate ester mineralization by microorganisms in these particular soils are clearly warranted. In addition to the mammalian formation and excretion of ester sulfate, soils are also capable of synthesizing these esters, and sulfate ester formation by soil microorganisms may represent an important mechanism for regulating the availability of SO_4^{2-} for the cycle. Again, insufficient data obviates an evaluation of the general applicability of this latter possibility, since much of this work (see section, Generation of choline sulfate) was confined to studies of the generation of a single sulfate ester, viz., choline sulfate. Although fungi are capable of forming this ester in large quantities, almost all of the ester is retained intracellularly, and thus it will be released only after cell lysis (see section, Generation of Choline Sulfate). On the other hand, bacteria of the genus *Pseudomonas* form and release this ester when SO_4^{2-} is growth limiting (64), and this may represent a mechanism for storing readily utilizable SO_4^{2-} in a form that will not alter the existing soil pH. The release of SO_4^{2-} from this ester need not necessarily be mediated only by microorganisms since plant roots (189, 191) and leaves (189) are capable of transporting and hydrolyzing (188) the ester.

Atmospheric Pollution

Atmospheric pollution represents a major source of SO_4^{2-} for inland aerobic soils (138, 150, 153), and seawater aerosols (138) contribute some of the SO_4^{2-} entering coastal soils. There is evidence that highly industrialized areas receive proportionally more sulfur from pollution than rural areas of low industrialization. For example, in coastal areas such as Norfolk, Va., 33.5 pounds (ca. 150.8 kg) of sulfur per acre per year were deposited in rainfall, whereas in a less industrialized area such as Halifax, Nova Scotia, only 12.5 pounds (ca. 56.3 kg) were deposited (150). The pollution-derived SO_4^{2-} in rain and snow (138, 150, 153) originates as a consequence of the oxidation (by a number of different mechanisms; see reference 138) of SO_2 and SO_3 which occur in air as pollutants. The results of studies by Freney and co-workers (90, 91) suggest that this SO_4^{2-} will be either used directly for plant growth or will be converted to ester sulfate (90) and later utilized for plant growth (91). In fallow soils or soils weeded before planting, exogenously derived SO_4^{2-} does not accumulate as such before utilization by growing plants. Freney et al. (90) found that $^{35}SO_4^{2-}$ was readily incorporated into HI-reducible S (see section, Ester sulfate) in soils of this type. Sulfur dioxide can also enter soils directly (51). Smith et al. (229) found that many agricultural soils sorb substantial amounts of this gas, but it is uncertain as to whether this SO_2 can be subsequently oxidized to SO_4^{2-}.

Sulfonates, Sulfamates, and the Sulfated-Thioglycosides

Apart from SO_4^{2-} returned to soils from atmospheric pollution, a review of factors generating SO_4^{2-} for the sulfur cycle would not be complete without a consideration of the metabolism of compounds that possess C—SO_3^- (sulfonate), N—SO_3^- (sulfamate), and the N—O—SO_3^- linkages. Sulfolipids and sulfocarbohydrates possessing the sulfonate linkage are widely distributed in green plants (18, 19, 36, 109, 134, 151, 272) and to a lesser extent in algae (226), protozoans (36, 37), bacterial membranes (18, 19, 148), and bacterial spores (22). Although a sulfonate has never been isolated from soil, it is obvious that these compounds (like compounds possessing the C—O—S linkage) may be easily released and possibly mineralized in this environment. This latter consideration is given some support by the isolation from soil of several *Flavobacterium* species that liberated SO_4^{2-} from a lipid sulfonate found in plant tissue (164). The existence of an analogous enzyme, capable of hydrolyzing a phosphonate linkage, was demonstrated recently in plants, insects, animals, and microorganisms (139). Moreover, many of the alkyl-benzene sulfonates, which arise in soils and waters as pol-

lutants, are readily degraded by microorganisms (see, e.g., references 115, 197, 198). The sulfated-thioglycosides (oxime *O*-sulfate esters) that possess the N—O—SO_3^- linkage are synthesized and released into soil by most, if not by all, members of the *Cruciferae* family of plants (142, 264). Once acted upon by a thioglycosidase of widespread occurrence in plants and fungi (53, 212), these esters can serve as sources of SO_4^{2-}. At present a great deal of confusion exists as to whether the subsequent release of SO_4^{2-} occurs enzymatically or by a nonenzymatic rearrangement; evidence for and against the involvement of a sulfohydrolase was considered in reviews by Roy and Trudinger (212) and by Dodgson and Rose (53). Irrespective of the mechanism involved, it is clear that these esters may represent a major source of SO_4^{2-} for soils possessing cruciferous plants. Unfortunately, information is not available regarding the quantity of this anion that is returned to soils from plant life.

Korn and Payza (144) and Dietrich (45, 46) demonstrated the existence of an enzyme in *Flavobacterium heparinum* (a soil isolate [203]) which liberated SO_4^{2-} from the sulfamate groups present in heparin. The occurrence of this sulfated polysaccharide in soil has already been discussed (see section, Origins of Soil Ester Sulfate).

S-Containing Amino Acids

A consideration of cystine and cysteine degradation as a source of SO_4^{2-} is necessary, since this may represent the major means of completing the sulfur cycle in aerobic soils that lack enzymes capable of oxidizing S^0. Various fungi (94, 182, 235-237) and bacteria (82, 236, 237) of soil origin converted cysteine and cystine to SO_4^{2-} aerobically, and Freney (84, 85) demonstrated that aerated soils also possessed enzymes capable of degrading these amino acids directly to SO_4^{2-}. In addition, the sulfur present in methionine was converted aerobically to this anion by a mixed population of soil microorganisms (111) and by *A. niger* (94). Possible pathways for the direct aerobic conversion of cysteine to SO_4^{2-} were considered in a review by Freney (87). The sulfur in this amino acid was also degraded aerobically to S^{2-} by *Escherichia coli* (143, 236) and by *P. vulgaris* (6, 143), but the mechanism for this transformation is not well understood. In more recent work, Swaby and Fedel (244) found that 41% of 56 Australian soils converted cystine to S^{2-}, but results of experiments designed to test the further oxidation of S^{2-} were not reported. Although S^{2-} can be oxidized nonenzymatically to S^0, only 14% of these soils rapidly oxidized S^0 to SO_4^{2-} (244), demonstrating that S^0 and cystine are not suitable precursors of SO_4^{2-} for these soils. With the exception of a recent but brief report (C. Hagedorn, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, I126, p. 137), similar published accounts of the ability of other soils to oxidize S^0 are unavailable. Although the application of S^0 is sometimes used to lower soil pH, it is not known to what extent S^0 oxidation acts as a source of SO_4^{2-} for soils in general. It is possible that Australian soils that are deficient in the ability to oxidize S^0 may depend largely on their reserve of ester sulfate (see Table 1) as a major source of SO_4^{2-}. Possible sources of this anion for well-drained, aerobic soils in general are summarized in Fig. 1.

PRACTICAL AND FUTURE CONSIDERATIONS

Although a great deal is known of parameters regulating the supply of SO_4^{2-} in anaerobic environments, much less is known of the analogous processes in aerobic soils, and yet aerobic soils are more extensively used for agriculture. Indeed, the question may be asked, does a cyclic transformation of sulfur always occur in aerobic soils? At present, there is insufficient information to answer this question even though an affirmative answer has been obtained for partially anaerobic environments (see, e.g., references 29, 129, 205).

Since aerobic soils provide the major source of the world's food, factors that generate SO_4^{2-} for these soils are not only related to an understanding of the sulfur cycle but also determine how much SO_4^{2-} will be available for plant growth and hence animal survival. Toward this end, Tisdale (259) stressed that a "greater knowlege of the factors affecting the release of sulfur (from organically bound ester sulfate) would be of immense value in predicting the supply of this element available to growing crops." Despite the SO_4^{2-} that is returned in the form of air pollution and ocean aerosols, sulfur deficiencies have occurred in inland aerobic soils (see, e.g., references 7, 20, 128, 244, 259). The belief held by some ecologists that sulfur is nonlimiting in this environment may now need to be reevaluated, especially if the proposed transfer and use of nitrogen-fixing genes to improve plant growth becomes a reality. In this connection, Postgate (207) stressed that in soils where nitrogen is not the nutrient limiting plant growth, then a limitation of sulfur as well as other elements could be expected if plant growth is intensified. In view of the continued use of fertilizers of limited sulfur content (20,

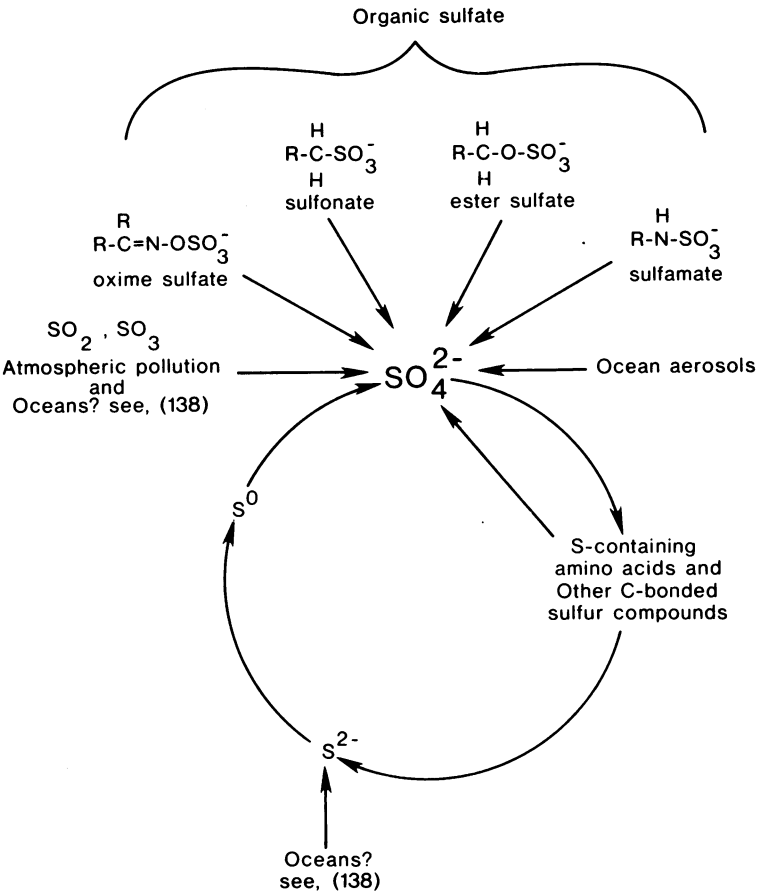

FIG. 1. *Sources of inorganic sulfate for aerobic, well-drained soils. SO_4^{2-}, inorganic sulfate; S-containing amino acids, cysteine, cystine and methionine; S^{2-}, sulfide; S^0, elemental sulfur.*

259), existing and proposed restrictions on SO_2-SO_3 pollution may mean that a greater future demand by plants will be made on the soil's reserve of ester sulfate rather than free SO_4^{2-}. This possibility is particularly pertinent in underdeveloped countries in which land is still largely fertilized with animal excreta.

It is clear that sulfate ester hydrolysis represents a source of SO_4^{2-} for soil, but it is impossible at this stage to evaluate the magnitude of this contribution. Apart from the elegant studies of Freney and co-workers, further investigations of the nature, formation, and release of SO_4^{2-} from organic sulfate need to be carried out not only for Australian soils but for aerobic soils throughout the world. Although it is clear that sulfur is present in this fraction as ester sulfate, the kind(s) of ester linkage(s) involved and other properties of the macromolecules comprising this fraction remain to be determined. Soil is decidely the most complex mixture on this planet not only from a microbiological but also from a physical and chemical viewpoint. Since it is impossible to control all the variables involved, it is believed that questions regarding the generation of SO_4^{2-} in this environment will not be totally answered by studies dealing entirely with the natural system. These investigations should be complemented by further studies of the localization of enzymes catalyzing the release of SO_4^{2-} and of factors governing the synthesis of these enzymes in microorganisms and plants grown or maintained in pure culture. These latter investigations should be directed toward determining the properties of the appropriate enzymes in microorganisms, isolated from soils that are known to contain high concentrations of ester sulfate. To date this has not been done, and yet the exhaustive studies by Tabatabai and Bremner of the sulfur status of Iowa soils represent an ideal foundation for such an investigation in the United

States. Considering the direction of future research on sulfur in agriculture, Tisdale (259) listed factors influencing mineralization and immobilization of sulfur in soils as having high priority second only to the definition of the pathways of sulfur metabolism in cells, and stated that "when this knowledge is translated into agricultural practice, it will contribute immeasurably to more efficient crop and animal production."

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