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Liberation of Sulfate from Sulfate Esters by Soils

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When incubated with acid, alkaline, and neutral soils, a variety of synthetic sulfate esters representing the various classes of these compounds was hydrolyzed by enzymes, probably of microbial origin. The appearance of sulfate in the soil water occurred immediately after introduction into the soils with some esters, whereas with others it occurred only after lag periods. Heat treatment destroyed the hydrolytic activity in the soils. The ester sulfate groups present in humic acid extracted from the soil appeared to be resistant to hydrolysis by a variety of sulfohydrolases extracted from bacteria and other organisms.

Sulfuric acid esters of various kinds are thought to play an important part in the metabolism of sulfur in soils (7, 8, 16, 17, 28) since appreciable quantities of sulfur are returned to the soil in this form in decaying organic matter and natural fertilizers. This arises from the widespread distribution of sulfate esters as structural components of plant and animal tissues and also as excretion products. The utilization of sulfate from these sources would involve the participation of a variety of degradative enzymes, including sulfohydrolases probably of microbial origin, which could liberate inorganic sulfate.

In support of this notion, several species of microorganisms, some of which are found in soils, have been shown to be capable of hydrolyzing various types of sulfate esters (31, 32). The latter studies have, however, been concerned with culturing microorganisms from soils and examining the sulfohydrolases present in harvested cells or in the culture medium. It has not been possible to deduce from this work what the fate of sulfate esters might be in the soil environment under natural conditions. Furthermore, if a wide variety of sulfohydrolases are active in the soil environment, it becomes necessary to explain the existence of ester sulfate groups that supposedly occur in the large organic soil colloids, humic and fulvic acids (18, 25). These are relatively stable molecules that could represent a storage form of sulfate.

The present study was undertaken to examine the fate of various types of sulfate esters when they remained in contact with undisturbed soil for various lengths of time and to determine the stability of the ester sulfate groups of humic acid towards various types of sulfohydrolases. The implication of the findings for better understanding both the soil sulfur cycle and the potential of the soil environment as a source of sulfohydrolases which may be useful analytical tools is emphasized.

MATERIALS AND METHODS

Soils. A Spodosol orthod, an Inseptisol ochrept, and a Mollisol rendoll soil were selected for study and obtained locally from Glamorgan and the Marlborough Downs, Wiltshire, U.K. Twelve random samples of the top 15 cm of the soils were collected, pooled, and sieved (2-mm mesh) while fresh. The pH of the soils was measured immediately after collection in a fresh soil-water mixture (1:2.5, wt/vol). The water content was determined after heating weighed samples of fresh soil to 110 C for 16 h. Samples were monitored for sulfohydrolase activity within 12 h of collection but for other purposes were stored at 2 C for up to 4 weeks.

Humic acid. Humic acid was extracted from 1 kg of fresh Inseptisol ochrept (equivalent to approximately 750 g of oven-dried material) by gently agitating with 4 liters of 0.5 M NaOH for 1 h. The insoluble material was separated by centrifuging at 3,000 × *g* for 1 h, and the supernatant was then acidified to pH 2.0 by the careful addition of 5 M HCl. After standing for 30 min, the precipitated humic acid was recovered by centrifuging at 3,000 × *g* for 1 h and dried by lyophilization to give fraction A (ash, 25.9%; water, 4.7%).

The acid was then dissolved in alkali as before, and the solution was clarified by centrifuging at 78,000 × *g* for 1 h. The humic acid was precipitated by acidification with HCl as before, and the whole procedure was repeated on the precipitate twice more. The final precipitate was then suspended in distilled water, dialyzed overnight, and lyophilized (fraction B: ash, 6.8%; water, 4.4%). A further deashing of fraction B was achieved by treatment with a hydrochloric acid-hydrofluoric acid mixture as described by Lowe (25). This yielded fraction C

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(ash, 0.3%; water, 2.1%). The yield was approximately 0.75% (wt/vol) with respect to the fresh soil. Attempts to deash the fraction further by treating again with hydrochloric acid-hydrofluoric acid yielded a material that had a lower sulfate content (fraction D, see Table 4). Only fractions A, B, and C were used for the enzyme studies.

Chemicals. Radioactively labeled sulfate esters were prepared from ^{35}S-labeled sulfuric acid (98%) or chlorosulfonic acid (Radiochemical Centre, Amersham, Bucks., U.K.) by the following methods: choline [^{35}S]sulfate, 13.6 μCi/mg (29); dipotassium 2-hydroxy-5-nitrophenyl [^{35}S]sulfate, 10.8 μCi/mg (15); potassium dodecyl [^{35}S]sulfate, 18.1 μCi/mg (2); potassium *p*-nitrophenyl [^{35}S]sulfate, 13.1 μCi/mg (11); potassium glucose [6-^{35}S]sulfate, 10 μCi/mg(24); potassium phenyl [^{35}S]sulfate, 15.8 μCi/mg(19); potassium L-tyrosine *O*-[^{35}S]sulfate, 3.7 μCi/mg(6). Potassium tyrosylglycine *O*-[^{35}S]sulfate (3.12 μCi/mg) was prepared by the method employed for L-tyrosine *O*-sulfate but substituting equimolar quantities of tyrosylglycine for L-tyrosine. Aqueous solutions (25 mM) of the esters were prepared, and the specific radioactivity of each was adjusted to 1.3 μCi/mg by adding the appropriate amount of 25 mM solution of homologous unlabeled material that had been prepared by the same method from unlabeled acid. Solutions were stored at −20 C until required.

Tyrosylglycine was obtained from Yeda Rehovot, Israel, and Oronite was from the Oronite Division, California Chemical Co., San Francisco, Calif. All other chemicals were purchased from B.D.H. Ltd., Poole, Dorset, U.K., and wherever possible were of AnalaR grade.

Enzyme preparations. A sample of alkylsulfohydrolase was prepared from *Pseudomonas* C12B (kindly provided by W. J. Payne, University of Georgia); cells were grown, and the sulfohydrolases were extracted as previously described (14).

Arylsulfohydrolases were prepared from three sources. A microbial enzyme was obtained from *Alcaligenes faecalis* NCIB 8734 by the method of Dodgson et al. (4) by dissolving 20 mg of the acetone-dried powder (powder B) in 20 ml of ice-cold 0.1 M phosphate buffer, pH 8.75. A crude preparation containing both arylsulfohydrolase and glycosulfohydrolase activity was obtained from the limpet *Patella vulgata* by the method of Dodgson and Spencer (9). A soluble extract of the acetone-dried limpet powder was obtained by homogenizing 1 g in 100 ml of ice-cold 0.5 M sodium acetate-acetic acid buffer, pH 5.5. The insoluble debris was removed by centrifuging at 79,000 × *g* for 30 min at 4 C.

The third source of arylsulfohydrolase was the digestive gland of the snail *Helix pomatia*. The enzyme was obtained in a partially purified state (stage 2) by the method of Dodgson and Powell (5). The preparation was diluted by mixing 1 ml of the extract with 200 ml of ice-cold 0.5 M sodium acetate-acetic acid buffer, pH 6.6, before use.

A glycosulfohydrolase was extracted from the visceral sac of the large periwinkle *Littorina littorea* and partially purified (stage 5) by the method of Lloyd (23). The enzyme preparation was obtained by dissolving 165 mg of the lyophilized powder in 22 ml of ice-cold 0.5 M tris(hydroxymethyl)aminomethane-acetic acid buffer, pH 5.5.

Paper chromatography and electrophoresis. One-dimensional descending chromatography was performed on Whatman no. 1 paper for 16 h at room temperature with butan-1-ol-acetic acid-water (4:1:2, by volume). Paper electrophoresis was conducted on Whatman no. 1 paper in 0.1 M sodium acetate-acetic acid buffer, pH 4.5, for 2 h at a potential gradient of 11 V/cm.

Detection and measurement of radioactivity. Radioactive components on paper chromatograms or electrophoretograms were detected by exposing to X-ray film (Ilford Industrial B) for periods of up to 4 weeks and also by scanning with a Packard radiochromatogram scanner (model 7200). The relative amount of radioactivity associated with each area was measured from the record of the scanner as described by Dodgson and Tudball (12). Total radioactivity in liquid samples was also determined by scintillation counting by the method of Dolly et al. (13).

Sulfohydrolase assay procedures. The sulfohydrolase activities of the preparations toward their normal assay substrates were determined before testing their activity toward humic acid. Arylsulfohydrolase activity was measured spectrophotometrically (10) using the colorimetric substrates and conditions described in Table 1. Alkylsulfohydrolase activity of the *Pseudomonas* C12B extract was determined by incubating 0.2-ml samples of the enzyme and potassium dodecyl [^{35}S]sulfate under the conditions described (Table 1). The reaction was stopped and deproteinization was achieved by heating at 100 C for 2 min, followed by centrifugation at 2,000 × *g* for 10 min. Liberated sulfate was estimated by electrophoresis and scanning as described by Dodgson and Tudball (12). The glycosulfohydrolase activity of the periwinkle extract was estimated as described in Table 1 using the barium chloride-gelatin method of Dodgson (3).

Determination of sulfohydrolase activity toward humic acid. The same conditions were used for the assay of activity of the various enzyme preparations toward humic acid. Reaction mixtures (20 ml) containing equal volumes of fraction C humic acid suspension (20 mg/ml) and sulfohydrolase preparations were incubated for 24 h under the appropriate conditions for each enzyme (Table 1). Samples (2.5 ml) were removed at 30-min intervals for up to 2 h and thereafter every 6 h. Organic acid and protein were precipitated by adjusting the pH to 1.5 with 5 M HCl. After freezing at −10 C and thawing, the suspended material was readily separated by centrifuging at 2,000 × *g* for 30 min. A 1.5-ml sample of the supernatant was lyophilized, and the solid residue was taken up in 0.1 ml of water for the determination of its inorganic sulfate content. Controls in which the humic acid and enzyme had been incubated separately were also prepared. Determinations of sulfate by the barium chloride-gelatin method of Dodgson (3) were made at 400 nm, where the absorption due to pigmented materials not precipitated by acidification did not interfere.

Soil analysis. The organic content of the soils was

derived (1) from carbon content estimations performed by the method of Walkley and Black (35). The total sulfur content of the soils was determined on 100 mg of the sieved, oven-dried (16 h at 110 C) sample by the procedure of Steinbergs et al. (30). The total sulfate (free inorganic sulfate plus ester sulfate) was estimated on similar samples by the method of Johnson and Nishita (20). The free inorganic sulfate was determined by the "difference method," which consisted of precipitating the sulfate as lead sulfate by the addition of alcoholic lead nitrate to an aqueous soil extract (22) and estimating the excess lead spectrophotometrically by the dithizonate method (34).

The results of the soil analysis are given in Table 2. The total sulfate content of the humic acid fractions was determined by the method of Johnson and Nishita (20).

Determination of sulfohydrolase activities of soils. Sieved, freshly collected soil (2 g) was lightly packed into each of 15 glass filter funnels (sintered-glass disk, 4 by 1 cm, porosity × 4; A. Gallenkamp & Co., Ltd., London EC2P 2ER, U.K.) at 2 C, and the labeled sulfate ester solution was then added to make the total soil water 5 mM with respect to the ester. The mixture was stirred thoroughly with a glass rod, and the tops of the filters were plugged with cotton wool. A large shallow tray containing water was placed within the incubator to humidify the atmosphere. Under these conditions weight changes of the sample due to water loss were very slight (1%) over 36 h at 28 C. Three tubes were taken after standing for 0, 6, 12, 24, and 36 h at 28 C, and 0.2 ml of water was stirred into the mixture. A sample of the soil water was then obtained by centrifuging the filter funnel within a tapered centrifuge tube, fitted with a rubber collar, at 850 × *g* for 15 min. Samples (50 μl) of the solution obtained were analyzed as described. The experiments were repeated using samples of soil that were autoclaved at 121 C for 15 min and cooled to room temperature prior to incubation with ester sulfates

RESULTS

Hydrolysis of sulfate esters in soils. Neither inorganic sulfate nor any of the sulfate esters could be completely recovered in the soil water, even when the analysis immediately followed mixing. The same effect was observed with sterilized soils. It was not possible to determine the extent of the adsorption for all possible mixtures of each of the esters and inorganic sulfate, but this was determined at one concentration. Inorganic sulfate and each of the esters were added as 25 mM solutions to soil samples as in the test experiments, followed by immediate analysis of the soil water using the method described. The experiment was repeated on sterilized soils. With the exception of the Inseptisol ochrept sample, both inorganic sulfate and most of the ester sulfates were adsorbed to the same extent (Table 3).

Precise determinations of the total hydrolysis

TABLE 1. *Conditions used to determine the activities of various sulfohydrolase preparations*

Sulfohydrolase prepn	Test substrate (potassium salts of:)	Buffer	Reactants (final concn)		Temp (C)	Rate of hydrolysis (μg of SO_4^{2-} released/h per ml of reaction mixture)
			Protein (mg/ml)	Substrate (mM)		
Alkylsulfohydrolase *Pseudomonas* $C_{12}B$	Dodecyl sulfate	0.05 M Tris-hydrochloride, pH 7.5[a]	5.4	5.0	30	62.5
Arylsulfohydrolase *Alcaligenes faecalis* NCIB no. 8734[b]	*p*-Nitrophenyl sulfate	0.1 M phosphate, pH 8.75	0.5	1.5	37	57.5
Patella vulgata	Nitrocatechol sulfate	0.5 M sodium acetate-acetic acid, pH 5.5	0.7	10.0	37	172.5
Helix pomatia	Nitrocatechol sulfate	0.5 M sodium acetate-acetic acid, pH 6.6	0.1	0.25	37	192.5
Glycosulfohydrolase *Littorina littorea*	Glucose 6-sulfate	0.5 M Tris-acetate, pH 5.5	3.8	3.0	37	214.0

[a] Tris, Tris(hydroxymethyl)aminomethane.
[b] NCIB, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, U.K.

TABLE 2. *Composition of the soils that were assayed*

Soil type	Sulfur content (μg/g)			pH	Water content (%)	Organic content (%)
	Total sulfur	Total sulfate	Inorganic sulfate			
Spodosol orthod	777	237	6.5	4.0	38.0	23.0
Inseptisol ochrept	613	258	5.4	6.2	28.5	8.4
Mollisol rendoll	665	240	0.7	8.4	24.0	6.8

TABLE 3. *Adsorption of inorganic and organic sulfate by soils*[a]

Soil type	Retention of sulfate ester (%)	Retention of inorganic sulfate (%)
Spodosol orthod	69	66
Inseptisol ochrept	71	93
Mollisol rendoll	61	61

[a] The quantity of [^{35}S]sulfate ester present in 10 μl of the 0-h samples of each soil water used in Fig. 1a to g was compared with the quantity present in 10 μl of the 25 mM sulfate ester solutions applied to the soils. Figures for NP^{35}S and NC^{35}S were not obtained because of their rapid breakdown, and choline sulfate was not tested with the Mollisol rendoll. Identical amounts of a 25 mM $K_2{}^{35}SO_4$ solution (0.5 μCi/mg) were added to the soils, and the recovery of [^{35}S]sulfate was similarly determined at zero time. Radioactivity was measured by liquid scintillation counting. Results are the average of three determinations on each of the sulfate esters.

occurring in the sample by measuring the situation in the soil water were, therefore, not possible, and, moreover, complete recovery of the adsorbed materials could not be achieved satisfactorily by elution with solutions of various ions. Measurement of the organic moieties liberated was similarly unsatisfactory as a means of determining the extent of hydrolysis since in most cases these were metabolized further. Measurements of the amount of ester and inorganic sulfate in the soil water at intervals were, however, made since this gave an indication of the rate at which sulfate became available from the various esters for further utilization as under natural circumstances.

It appears that the esters were probably hydrolyzed by microorganisms or particle-adsorbed enzymes in the experimental soils since sterilization prior to incubation with ester sulfates destroyed the ability of the soils to hydrolyze all of the esters during the experimental period of incubation. Rates of sulfate release differed markedly, however. Although data are not presented, potassium dodecyl sulfate was hydrolyzed most rapidly in all three soils, nearly all the sulfate being liberated within 1 h. The ratio of ester to inorganic sulfate in the soil water could not be determined in this case since the ester was completely adsorbed onto the soil particles immediately. This did not prevent the measurement of the rate of inorganic sulfate release into the soil water, however. Potassium *p*-nitrophenyl sulfate and dipotassium nitrocatechol sulfate were also hydrolyzed extremely rapidly in all three soils (Fig. 1c and d), some hydrolysis having occurred within the time taken to separate the soil water without preincubation. In further experiments with Inseptisol ochrept, hydrolysis was complete in 2 h. Other esters were hydrolyzed more slowly, and several were minimally affected when incubated with Spodosol, pH 4.0 (Fig. 1a,b,e,f, and g).

The initial rate of hydrolysis of phenyl sulfate (Fig. 1b) appeared slower than that of the other arylsulfates (Fig. 1c and d). Hydrolysis of the additional esters (Fig. 1a,b,e,f, and g) appeared to require lag periods before hydrolysis commenced, although lag times were decreased as soil alkalinity increased. G6^{35}S was subjected to complex catabolism by soil microorganisms and enzymes (Fig. 2A). Within 24 h the parent compound was dissimilated, but four sulfur-bearing intermediates were accumulated in addition to inorganic sulfate. TG^{35}S underwent apparently simple cleavage before ester sulfate was removed. The dipeptide was hydrolyzed to yield glycine and a component that cochromatographed with TO^{35}S, and it was from the latter monomer that sulfate was removed.

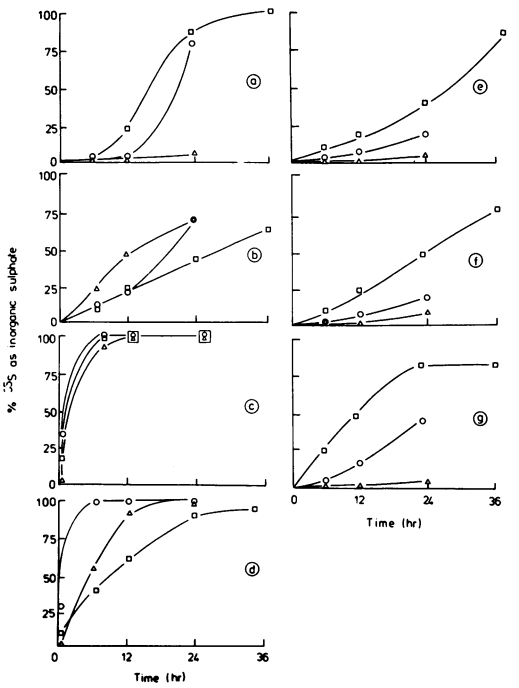

FIG. 1. *Time courses of sulfate ester hydrolysis in soils of three types. (a) Choline [^{35}S]sulfate (C^{35}S); (b) potassium phenyl [^{35}S]sulfate (P^{35}S); (c) potassium p-nitrophenyl [^{35}S]sulfate (NP^{35}S); (d) dipotassium nitrocatechol [^{35}S]sulfate (NC^{35}S); (e) potassium L-tyrosine O-[^{35}S]sulfate (TO^{35}S); (f) potassium tyrosylglycine O-[^{35}S]sulfate (TG^{35}S); (g) potassium glucose [6-^{35}S]sulfate (G6^{35}S). Symbols: △, Spodosol orthod, pH 4.0; ○, Inseptisol ochrept, pH 6.2; □, Mollisol rendoll, pH 8.4.*

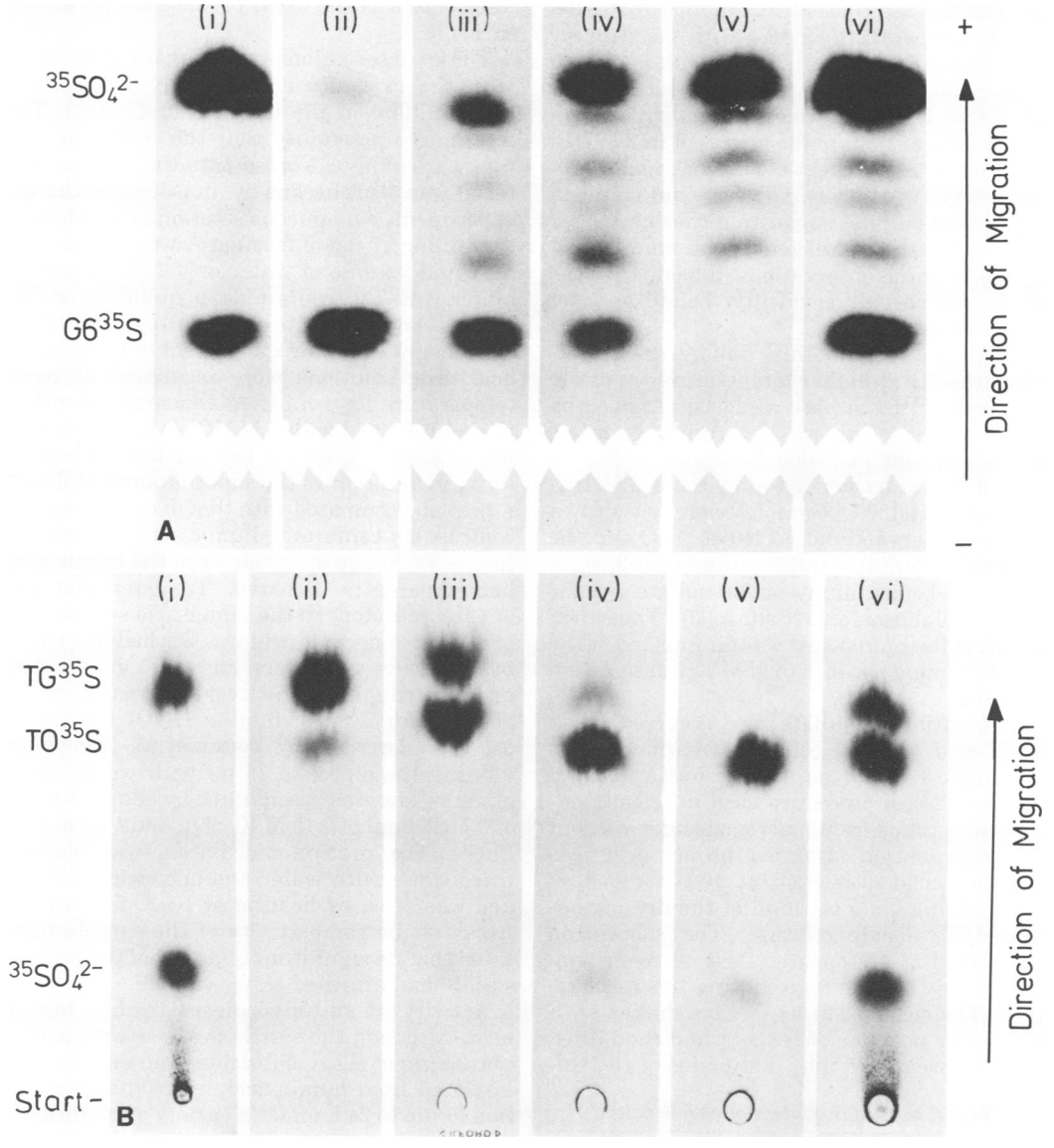

FIG. 2. *Sulfated intermediates of $G6^{35}S$ and $TG^{35}S$ degradation in soils. (A) Radioautogram of an electrophoretogram of the products of $G6^{35}S$ catabolism in the Mollisol rendoll soil: (i) authentic $G6^{35}S$ plus inorganic [^{35}S]sulfate; (ii) 0 h; (iii) 6 h; (iv) 12 h; (v) 24 h; (vi) (i) plus (v). (B) Radioautogram of a chromatogram of the products of $TG^{35}S$ catabolism in the Inseptisol ochrept soil; (i) authentic $TG^{35}S$ plus inorganic [^{35}S]sulfate; (ii) 0 h; (iii) 6 h; (iv) 12 h; (v) 24 h; (vi) (i) plus (v).*

Sulfur content of humic acid. Although part of the sulfur in humic acid is known to be ester sulfate, it was necessary to establish the characteristics of the sample used in this work before attempting to detect the sulfate ester groups and characterize them enzymatically. Humic acid was chosen over fulvic acid or other soil organic fractions because of the relative ease of extracting reasonable yields from soils. Humic acid has a higher sulfate content than fulvic acid, irrespective of the method of extraction and fractionation (18). The three fractions (A, B, and C) of humic acid used in this work all contained roughly the same amount of sulfur, and approximately 50% of this appeared to be mainly sulfate (Table 4) since it was readily reducible by the method of Johnson and Nishita (20). It appeared that the sulfate was relatively stable in acid solution at mild temperatures. It can be seen that the procedure employed to

diminish the ash content, including incubation at room temperature for 40 h with the strongly acidic HCl-HF solution, did not result in a notable decrease in the sulfate content of the material (Table 4). Sulfate release similarly did not occur in suspensions of the acid kept in 2 M HCl at 4 C for 96 h. A repeat of the deashing procedure to give fraction D, however, did result in some loss of sulfate, suggesting that prolonged treatment with this reagent had a mild labilizing effect. These observations indicate that the sulfate was largely covalently bound and not just adsorbed.

In agreement with this, sulfate release occurred readily at higher temperatures in dilute acid, and all the sulfate could be removed by such treatment. This was determined in a further experiment in which a sample (50 mg) of fraction D was suspended in 30 ml of 2 M HCl and heated at 100 C. Samples were withdrawn at 5-min intervals and cooled to 4 C, and the acid was precipitated by centrifuging at 2,000 × *g* for 1 h before analysis for sulfate by the method of Johnson and Nishita (20). The sulfur content of the humic acid was diminished at the rate of 0.9 mg/g per min to give 90% desulfation in 30 min.

A liberation of sulfate also occurred when humic acid was subjected to treatment with methanolic HCl by the method of Kantor and Schubert (21), a procedure used to release sulfate ester groups from macromolecules without their degradation. Isolated humic acid was dried in vacuo over P_2O_5 at 40 C for 8 days before adding 0.4 g to 70 ml of the dry methanol-acetyl chloride mixture. The suspension was shaken in a stoppered flask at room temperture for 24 h before decanting the spent reagent. The suspension was again shaken with two further portions of reagent and then dried in vacuo (yield, 115 mg). Estimates of the sulfate content of the dried product are presented in Table 5.

TABLE 4. *Sulfur content of humic acid*[a]

Humic acid prepn	Sulfur content		
	Total (mg/g)	Total sulfate (mg/g)	Total sulfate (% of total S)
Fraction A	10.2	5.0	49
Fraction B	10.3	5.2	50.5
Fraction C	10.1	5.0	39.5
Fraction D	8.2	2.8	34

[a] The total sulfur and sulfate content of the acid was determined by substituting lyophilized organic material (10 mg) for soil in the systems described in the text. Results are the average of three determinations and are corrected for ash and water content. Fractions A, B, C, and D were successive samples obtained in the deashing process described in the text.

These observations were again consistent with the existence of covalently bound (i.e., ester) sulfate in the isolated humic acid. The alternative possibility that the inorganic sulfate was simply adsorbed onto the organic material was also checked by an isotope-exchange experiment. An aqueous solution of humic acid containing 17 mg of fraction A was mixed with an equal volume of water or 0.2 M phosphate buffer, pH 7.0, containing a quantity of ^{35}S-labeled sodium sulfate representing eight times the amount of sulfate ion present in the organic acid. The solutions were incubated at room temperature for 1 h before adjusting the pH of the solution to 2 with 5 M HCl. After centrifuging at 2,000 × *g* for 1 h the amount of radioactivity in a sample of the supernatant was determined and compared with that of corresponding controls not containing humic acid. No adsorption or exchange of sulfate onto the humic acid had apparently occurred. To determine the amount of isotope in the humic acid sulfate, the precipitated organic acid was washed five times by successively resuspending in 10 ml of water and centrifuging. The residue was then digested with 2 ml of fuming HNO_3 for 4 h at 360 C as described by Young et al. (36). After diluting the digest to 15 ml with water, inorganic sulfate was precipitated by adding 5 ml of 4 M HCl, 3 ml of 0.15 M K_2SO_4, and 4 ml of 10% $BaCl_2$. The precipitated $BaSO_4$ was washed three times with water and once with acetone and was dried by heating at 100 C for 30 min. Assay of the radioactivity of the sample indicated that no significant quantity of the labeled sulfate had attached to the sample.

Activity of sulfohydrolases toward humic acid. Although the sulfohydrolases were active toward their assay substrates, no sulfate was liberated from humic acid, even after incubation for up to 24 h under a variety of conditions.

DISCUSSION

Several types of sulfohydrolase, probably of microbial origin, were detected within the soil

TABLE 5. *Effect of methanolic HCl on the sulfur content of humic acid*[a]

Material	Sulfur content		
	Total (mg/g)	Total sulfate (mg/g)	Total sulfate (% of total S)
Untreated acid	10.4	4.9	47
Treated acid	8.9	3.2	36

[a] Results are the average of three determinations and are corrected for ash and water content.

environment. The absolute activity of these enzymes could not be determined since the situation was undoubtedly influenced by complex changes in the adsorption pattern of the unchanged esters and liberated inorganic sulfate as the incubation proceeded. However, changes in the appearance of sulfate and the concomitant disappearance of sulfate ester in the soil water could be observed. The relative rates of these changes for the various esters studied can be compared and can be considered to be due in part at least to the rate of sulfate hydrolysis, which in turn might be related in certain cases to enzyme induction in soil microorganisms. The rapidity with which they were degraded indicated that KD^{35}S, NC^{35}S, and NP^{35}S were hydrolyzed by enzymes already present in the microbial cells or adsorbed onto the colloids of each of the soil types. Other esters (C^{35}S, G6^{35}S, TG^{35}S, and TO^{35}S) were not hydrolyzed immediately, indicating the possible necessity for induction of dissimilatory enzyme synthesis in the soil microorganisms. The existence of enzyme systems other than sulfohydrolases was also detected. It appeared that G6^{35}S was degraded to at least four sulfated products and further that TG^{35}S was first slowly converted into another metabolite (probably into TO^{35}S) by peptidase action before desulfation (Fig 2A and B). It is thus not surprising that naturally occurring low-molecular-weight sulfate esters do not accumulate in soils, although the possibility remains that traces have not been observed due to the lack of suitable means of extraction and detection.

Ester sulfate, often generally referred to as reducible sulfate (16, 18, 25), was, however, shown to be present in the humic acid extracted from one of the soils used in this study, thus confirming the observations of earlier workers. Unfortunately, there is no specific test for the ester sulfate group, and its nature can only be inferred from the hydrolytic effect of various reagents, including 2 M HCl (100 C), methanolic HCl (room temperature), and HI (under reflux). The action of hot dilute HCl produces sulfate ions from sulfate esters (and more slowly from some sulfonates) but not from organic sulfides, sulfones, or sulfoxides. Under relatively mild conditions, sulfonates can generally be considered to be completely stable (28). Methanolic HCl will not attack sulfonate groups (33) but will attack the C-O-S linkage of sulfate esters. Reducing mixtures containing HI have been shown to produce H_2S only from ester and inorganic sulfate and organic sulfites (e. g., dimethyl sulfite; see T. H. Arkley, Ph.D. thesis, Univ. of California, Berkley, 1961, and unpublished data, our laboratory). Thus, the effect of these reagents on the isolated humic acid observed in the present study are consistent with the existence of sulfate ester groups in the material.

We were not able to determine whether the sulfate groups in the humic acid was alkyl-, aryl-, or carbohydrate esters because of their refractiveness to the various sulfohydrolase preparations. Indeed, the inactivity of the sulfohydrolases toward humic acid might suggest at first glance that sulfate ester groups are not present in the material, but other sulfated macromolecules (e. g., chondroitin sulfates, heparin; see references 7, 8) are resistant to enzymatic desulfation and require breakdown into low-molecular-weight subunits before desulfation occurs. The degradation of humic acid or other soil organic polymers apparently does occur slowly and is almost certainly carried out by microbial, multienzyme catalyzed sequences, producing low-molecular-weight intermediates such as benzoquinone, 2-methyl 1,4-naphthoquinone, salicyl alcohol, and salicylaldehyde (26, 27). Desulfation of humic acid could result from a sequential attack by depolymerizing and desulfating enzymes. Further studies along these lines would seem worthwhile since the existence of such a mechanism could indicate that the sulfohydrolases have an important role in releasing bound sulfate from storage in soil colloids for microbial and plant utilization.

In any event, the existence of a wide variety of interesting and useful sulphohydrolases in the soil environment is indicated. It can be safely concluded that any naturally occurring sulfate ester returned to the soil will yield inorganic sulfate in the soil water within a relatively short time. Moreover, it seems that these sources of enzymes could yield a number of valuable analytical tools for structural studies on sulfate esters.

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LITERATURE CITED

1. **Broadbent, F. E.** 1965. Organic matter, p. 1397-1400. *In* C. A. Black (ed.), Methods of soil analysis. American Society of Agronomy, Madison, Wis.
2. **Denner, W. H. B., A. H. Olavesen, G. M. Powell, and K. S. Dodgson.** 1969. The metabolism of potassium dodecyl [^{35}S]sulphate in the rat. Biochem. J. 111:43-51.
3. **Dodgson, K. S.** 1961. Determination of inorganic sulphate in studies on the enzymatic and non-enzymatic hydrolysis of carbohydrate and other sulphate esters. Biochem. J. 78:312-319.
4. **Dodgson, K. S., T. Melville, B. Spencer, and K. Wil-**

liams. 1954. Studies on sulphatases. 8. The arylsulphatase of a strain of *Alcaligenes metalcaligenes* isolated from inter-tidal mud. Biochem. J. 58:182-187.

5. **Dodgson, K. S., and G. M. Powell.** 1959. Studies on sulphatases. 27. The partial purification and properties of the arylsulphatase of the digestive gland of *Helix pomatia*. Biochem. J. 73:672-679.
6. **Dodgson, K. S., G. M. Powell, F. A. Rose, and N. Tudball.** 1961. Observations on the metabolism of tyrosine O-[^{35}S]sulphate in the rat. Biochem. J. 79:209-213.
7. **Dodgson, K. S., and F. A. Rose.** 1970. Sulfoconjugation and sulfohydrolysis, p. 239-325. *In* W. H. Fishman (ed.), Metabolic conjugation and metabolic hydrolysis, vol. 1. Academic Press Inc., New York.
8. **Dodgson, K. S., and F. A. Rose.** 1975. Sulfohydrolases, p. 359-431. *In* D. M. Greenberg (ed.), Metabolic pathways, vol. 7. Metabolism of sulfur compounds. Academic Press Inc., New York.
9. **Dodgson, K. S., and B. Spencer.** 1953. Studies on sulphatases. 4. Arylsulphatase and β-glucuronidase concentrates from limpets. Biochem. J. 55:315-320.
10. **Dodgson, K. S., and B. Spencer.** 1957. Assay of sulfatases, p. 211-255. *In* D. Glick (ed.), Methods of biochemical analysis, vol. 4. Interscience Publishers, New York.
11. **Dodgson, K. S., and N. Tudball.** 1960. The metabolic fate of the ester sulphate group of potassium *p*-nitrophenyl [^{35}S]sulphate. Biochem. J. 74:154-159.
12. **Dodgson, K. S., and N. Tudball.** 1961. Enzymic desulphation of L-serine O-[^{35}S]sulphate; the intracellular localization of the enzyme. Biochem. J. 81:68-71.
13. **Dolly, J. O., C. G. Curtis, K. S. Dodgson, and F. A. Rose.** 1971. Metabolism of sodium oestrone [^{35}S]sulphate in the rat. Biochem. J. 123:261-266.
14. **Fitzgerald, J. W., and W. J. Payne.** 1972. Induction in a *Pseudomonas* species of sulphatases active on short chain alkylsulphates. Microbios 5:87-100.
15. **Flynn, T. G., K. S. Dodgson, G. M. Powell, and F. A. Rose.** 1967. The metabolism of dipotassium 2-hydroxy-5-nitrophenyl [^{35}S]sulphate, a substrate for the lysosomal arylsulphatases A and B. Biochem. J. 105:1003-1012.
16. **Freney, J. R.** 1961. Some observations on the nature of the organic sulphur compounds in soil. Aust. J. Agric. Res. 12:424-432.
17. **Freney, J. R.** 1967. Sulphur-containing organics, p. 229-259. *In* A. D. McLaren and G. H. Peterson (ed.), Soil biochemistry, vol. 1. Marcel Dekker, New York.
18. **Freney, J. R., G. E. Melville, and C. H. Williams.** 1969. Extraction, chemical nature, and properties of soil organic sulphur. J. Sci. Food Agric. 20:440-445.
19. **Hawkins, J. B., and L. Young.** 1954. Biochemical studies of toxic agents. 5. Observations on the fate of ^{35}S-labelled arylsulphuric acids following their administration to the rat. Biochem. J. 56:166-170.
20. **Johnson, C. M., and Nishita, H.** 1952. Microestimation of sulphur. Anal. Chem. 24:736-742.
21. **Kantor, T. G., and K. Schubert.** 1957. A method for the desulfation of chondroitin sulfate. J. Am. Chem. Soc. 79:152-153.
22. **Little, I. P., R. Reeve, G. M. Proud, and A. Lulham.** 1969. Determination of microgramme quantities of dissolved sulphate in surface waters, soils and plants. J. Sci. Food Agric. 20:673-676.
23. **Lloyd, A. G.** 1966. Glycosulfatases from molluscs, p. 670-677. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
24. **Lloyd, A. G., P. J. Large, M. Davies, A. H. Olavesen, and K. S. Dodgson.** 1968. The glycosulphatase of *Trichoderma viride*. Biochem. J. 108:393-399.
25. **Lowe, L. E.** 1969. Distribution and properties of organic fractions in selected Alberta soils. Can. J. Soil Sci. 49:129-141.
26. **Mathur, S. P.** 1971. Characterization of soil humus through enzymatic degradation. Soil Sci. 111:147-157.
27. **Mathur, S. P., and E. A. Paul.** 1967. Partial characterization of soil humic acids through biodegradation. Can. J. Microbiol. 13:581-586.
28. **Roy, A. B., and P. A. Trudinger.** 1970. The biochemistry of inorganic compounds of sulphur. Cambridge University Press, London.
29. **Schmidt, E.** 1940. Uber Cholin, Neurin and verwandte Verbindungen. Justus Liebigs Ann. Chem. 337:37-121.
30. **Steinbergs, A., O. Iismaa, J. R. Freney, and N. J. Barrow.** 1962. Determination of total sulphur in soil and plant material. Anal. Chim. Acta 27:158-164.
31. **Tabatabai, M. A., and J. M. Bremner.** 1970. Arylsulfatase activity of soils. Soil Sci. Soc. Am. Proc. 34:225-229.
32. **Tabatabai, M. A., and J. M. Bremner.** 1970. Factors affecting soil arylsulfatases. Soil Sci. Soc. Am. Proc. 34:427-429.
33. **Tipson, R. S.** 1953. Sulfonic esters of carbohydrates, p. 107-215. *In* C. S. Hudson and M. L. Wolfrom (ed.), Advances in carbohydrate chemistry, vol. 8. Academic Press Inc., New York.
34. **Vogel, A. I.** 1961. A text-book of quantitative inorganic analysis including elementary instrumental analysis. Longmans, London.
35. **Walkley, A., and I. A. Black.** 1934. An examination of the Degtareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Sci. 37:29-38.
36. **Young, L., M. Edson, and J. A. McCarter.** 1949. The measurement of radioactive sulphur [^{35}S] in biological materials. Biochem. J. 44:179-185.