PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Mechanisms of Sulphate Activation and Transfer

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Mechanisms of sulphate activation and transfer

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SUMMARY

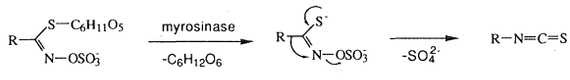
Sulphation of natural products is a widespread phenomenon. Inorganic sulphate is transported into cells and activated by ATP sulphurylase, an enzyme that has been studied by kinetic and stereochemical methods. It has been shown that the enzyme catalyses the displacement of inorganic pyrophosphate by inorganic sulphate from P_α of ATP by a direct 'in line' mechanism. The adenosine 5′-phosphosulphate formed is then phosphorylated at the 3′ position by APS kinase to give 3′-phosphoadenosine 5′-phosphosulphate, the common sulphating species in biology.

A general strategy for the synthesis of chiral [$^{16}O^{17}O^{18}O$]-sulphate esters has been established and a method developed for their stereochemical analysis by using Fourier Transform Infrared Spectroscopy. The stereochemical course of an aryl sulphotransferase from *Aspergillus oryzae* has been shown to proceed with retention of configuration at sulphur, supporting a ping pong type mechanism with a sulpho-enzyme intermediate on the reaction pathway.

INTRODUCTION

Sulphate half esters are found among all the major classes of natural products, i.e. nucleotides, peptides and proteins, polysaccharides, steroids and lipids. Since the classical study of Baumann (1876) well over a century ago, in which phenol administered to a patient was excreted as phenyl sulphate it has been clear that sulphation is used as a detoxification mechanism. Sulphation, however, plays other important functional roles among natural products. A number of secreted peptides and proteins (e.g. fibrinogen, fibrinopeptide A and B, cholecystokinin, hirudin, etc.) possess sulphated tyrosine residues. Thus fibrinogen, a soluble plasma protein, on proteolysis with thrombin releases fibrinopeptide A (18 residues) and B (20 residues), and the fibrin monomers formed spontaneously associate into insoluble fibres. Fibrinopeptides A and B both contain a sulphated tyrosine residue as well as aspartate and glutamate residues. The presence of these regions of high negative charge in fibrinogen prevents aggregation. Interestingly, the anticoagulant hirudin produced by the medicinal leech (*Hirudo medicinalis*), is a 65-residue peptide containing a negatively charged C-terminus, which includes a sulphated tyrosine residue at position 63. Hirudin is a potent inhibitor of thrombin and so prevents blood clotting at the site of incision thus allowing blood to be drawn freely. A recent report on the X-ray structural analysis of the complex of recombinant hirudin and human α-thrombin shows that the negatively charged tail binds at a highly positively charged region of thrombin remote from the active site (Rydel *et al.* 1990). The sulphated polysaccharide heparin also binds to this site in α-thrombin. The sulphate group found in proteoglycans (e.g. chondroitin sulphate, dermatan sulphate, etc.) ensures that the polysaccharide chains are separated and in extended conformations because of electrostatic repulsion. The function of these molecules appears to be to create a gel around cells with the ability to disperse shock. They are found in cartilage, arterial walls and connective tissue (Schubert & Hamerman 1968). Sulphate esters of steroids are important intermediates in the biosynthesis of steroidal hormones (e.g. progesterone, estrone, androstenolone, cortisone, etc.) (Bernstein & Solomon 1970). Steroid sulphatase deficiency leads to excretion of large amounts of steroidal sulphates leading to problems in pregnancy and childbirth (Shapiro *et al.* 1977). Sulpholipids are found in place of phospholipids in the membranes of some bacteria, clearly showing that sulphate monoesters can play a similar role to phosphate monoesters.

A particularly interesting function of sulphate esters is found in the mustard oil glycosides (e.g. sinigrin) present in *Brassica* species. When the thioglycoside bond is cleaved by myrosinase the sulphated thiohydroxamate undergoes a spontaneous Lossen-type rearrangement to give a volatile isothiocyanate, which is responsible for the characteristic biting taste and pungent odour of ground mustard seeds (Kjaer 1960, 1961).



R = allyl for sinigrin.

The pre-emergent herbicide 2-(2,4-dichlorophenoxy)ethyl sulphate (Crag herbicide, also known as Herbon) is inactive until the sulphate ester is hydrolysed by sulphatases produced by soil microorganisms,

in particular *Pseudomonas* species. The sulphate group makes the pro-herbicide water soluble and therefore easy to apply as well as ensuring a slow release of the active agent (Lillis *et al.* 1983).

ACTIVATION OF INORGANIC SULPHATE

Inorganic sulphate is used in the biosynthesis of sulphate half esters, but this inert, water soluble divalent ion has to be transported into cells and then enzymically activated. The structure of the sulphate binding protein that actively transports sulphate ions into *Salmonella typhimurium* has recently been determined at 2 Å† resolution (Pflugrath & Quiocho 1988). Although inorganic sulphate is an exceedingly poor nucleophile in aqueous solution, it behaves as such in the activation process. Robbins & Lipmann (1956, 1957, 1958) first isolated biologically activated sulphate and correctly characterized it as the nucleotide 3′-phosphoadenosine 5′-phosphosulphate (PAPS). It was subsequently synthesized by Baddiley *et al.* (1957).

3′-Phosphoadenosine 5′-phosphosulphate is synthesized enzymically from adenosine 5′-triphosphate and inorganic sulphate. ATP sulphurylase catalyses the first step in the synthesis in which adenosine 5′-phosphosulphate (APS) is formed.

$$ATP^{4-} + SO_4^{2-} + H^+ \rightleftharpoons APS^{2-} + PP_i^{3-} \rightarrow 2P_i.$$

The equilibrium constant for the formation of APS however is very unfavourable, $\Delta G_0'$ being about +9.5 kcal mol^{-1} at 5 mM Mg^{2+}, 25 °C and pH 7 (Robbins & Lipmann 1958; Akagi & Campbell 1962). As $\Delta G_0'$ for the hydrolysis of ATP to AMP and PP_i under the same conditions is −10 kcal mol^{-1} (Gwynn *et al.* 1974), $\Delta G_0'$ for hydrolysis of the phosphosulphate anhydride bond must be approximately −19.5 kcal mol^{-1}. Because of the unfavourable $\Delta G_0'$ for the formation of APS, if no other reactions were involved the concentration of the activated sulphate would be very low. The liberated inorganic pyrophosphate, however, is hydrolysed by inorganic pyrophosphatase, and the free energy locked in this phosphoanhydride bond released. $\Delta G_0'$ for this reaction is about −4.5 kcal mol^{-1} in the presence of excess Mg^{2+} at pH 7.

The third enzyme involved in the biosynthesis of PAPS is APS kinase which uses another molecule of ATP to phosphorylate the 3′-hydroxy group of APS. $\Delta G_0'$ for breaking the phosphoanhydride bond is between −4 and −5 kcal mol^{-1} bringing the overall equilibrium constant for the formation of PAPS from inorganic sulphate close to unity.

$$APS^{2-} + ATP^{4-} \rightleftharpoons PAPS^{4-} + ADP^{3-} + H^+.$$

In bacteria and fungi the activated sulphate group in PAPS can be reduced by NADPH first to inorganic sulphite with the enzyme PAPS reductase and then to hydrogen sulphide with sulphite reductase which is used for the biosynthesis of divalent sulphur containing compounds. PAPS is the common sulphating agent in biology.

† 1 Å = 10^{-10} m = 10^{-1} nm.

ATP SULPHURYLASE

ATP sulphurylase has been extensively studied. Initial velocity, product inhibition, dead-end inhibition, and isotope exchange studies have shown that ATP sulphurylase follows an ordered sequential kinetic mechanism, MgATP adding before sulphate and $MgPP_i$ dissociating before APS (Wilson & Bandurski 1958; Stadtman 1973; Tweedie & Segel 1971; Shoyab *et al.* 1972; Farley *et al.* 1976, 1978). MgATP analogues (ATP, CaATP, CrATP, or Mg αβ-methylene ATP), however, were shown to be incapable of inducing the binding of sulphate, and so it was concluded that either the structural requirements for the metal ion-nucleotide complex are so stringent that only MgATP can induce the conformational change that forms the sulphate binding site, or the mere binding of a metal ion-nucleotide complex is insufficient to promote the binding of sulphate. The latter explanation was favoured, with the implication that the MgATP must be catalytically cleaved to E-AMP. $MgPP_i$ before the sulphate binding site formed, and that the adenylated enzyme then bound inorganic sulphate and reacted with it. This conclusion, however was shown by stereochemical methods to be untenable as displacement at P_α of ATP by sulphate ion catalysed by ATP sulphurylase proceeds with inversion of configuration at phosphorus (Bicknell *et al.* 1982). Since our work was reported, Seubert *et al.* (1983, 1985) have shown that if ATP sulphurylase is rigorously purified the earlier observations, which had led to the proposal of an adenylated enzyme intermediate, could not be repeated. The kinetic and stereochemical evidence now clearly indicate that the activation of inorganic sulphate catalysed by ATP sulphurylase occurs by a direct 'in line' displacement at P_α of ATP.

SYNTHESIS AND STEREOCHEMICAL ANALYSIS OF CHIRAL [^{16}O,^{17}O,^{18}O] SULPHATE ESTERS

To study the stereochemical course of sulphuryl transfer reactions a general strategy was needed for the synthesis of chiral [^{16}O,^{17}O,^{18}O]sulphate monoesters of known absolute configuration as well as a method for their stereochemical analysis.

The general strategy for synthesis was to take a chiral 1,2- or 1,3-diol, convert it into the diastereoisomeric cyclic sulphites with [^{18}O]thionyl chloride and then oxidise the sulphite esters to the cyclic sulphate diesters with ruthenium [^{17}O]tetroxide. Regiospecific ring opening of the cyclic [^{17}O,^{18}O]sulphate diesters should give chiral [^{16}O,^{17}O,^{18}O]sulphate monoesters of known absolute configuration (Lowe & Salamone 1984). Nuclear magnetic resonance (N.M.R.) spectroscopy, which had been successfully used for the analysis of chiral [^{16}O^{17}O^{18}O]phosphate monoesters (Lowe 1983), did not commend itself for the analysis of chiral [^{16}O,^{17}O,^{18}O]sulphate monoesters as none of the isotopes of sulphur possesses a nuclear spin quantum number of $\frac{1}{2}$. Moreover, the exocyclic oxygen atoms in a cyclic sulphate diester cannot be alkylated to render

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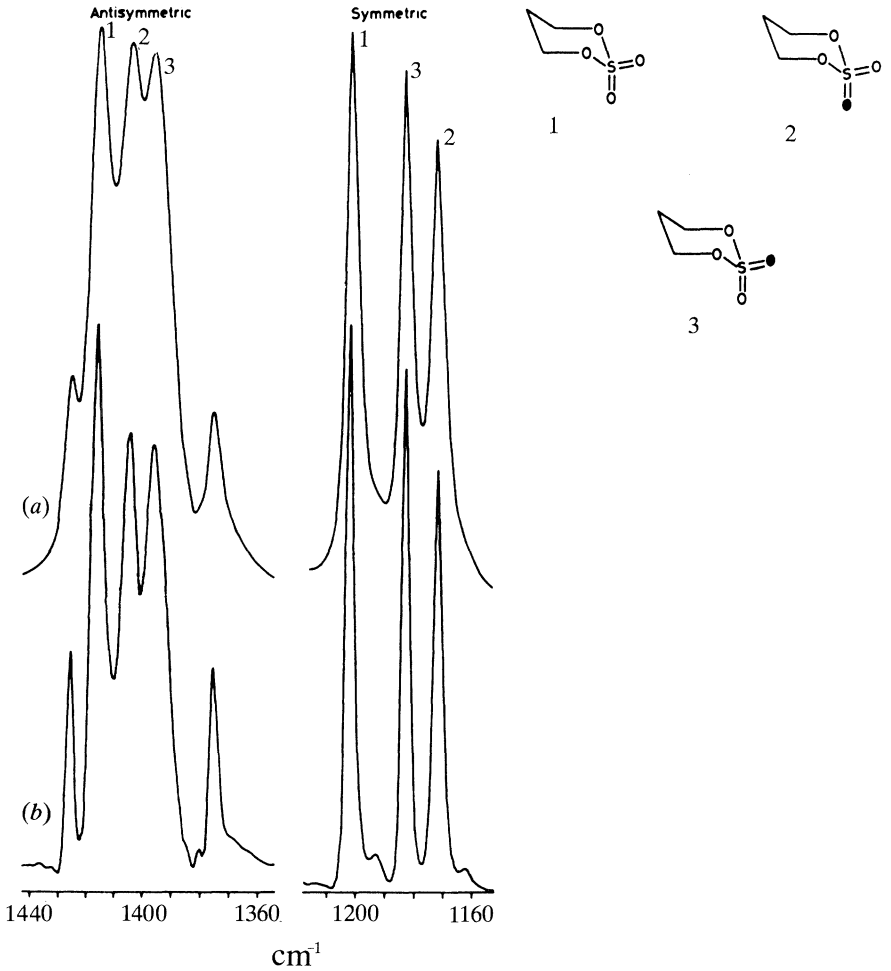


Figure 1. (*a*) The FTIR spectrum of the antisymmetric and symmetric $> SO_2$ stretching region of 2,2-[^{18}O]dioxo-1,3,2-dioxathiane and (*b*) the same spectrum after deconvolution with an enhancement factor of 1.68 and line-width at half height of 5 cm^{-1} for the antisymmetric $> SO_2$ stretching region and an enhancement factor of 1.50 and line-width at half height of 4 cm^{-1} for the symmetric $> SO_2$ stretching region. The spectra were measured on a Perkin-Elmer 1750 FTIR spectrometer at a resolution of 1 cm^{-1}.

Table 1. *The effect of oxygen isotopic substitution on the symmetric and antisymmetric* $> SO_2$ *stretching frequencies* (cm^{-1}) *of (4R)-4-methyl-2,2-dioxo-1,3,2-dioxathianes*

(Δ is the isotope shift from the $> SO_2$ frequency.)

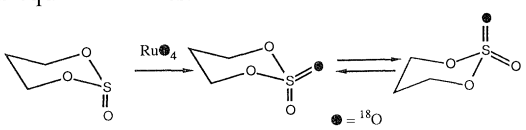
isotope		symmetric stretching frequency	Δ for symmetric stretching mode	antisymmetric stretching frequency	Δ for antisymmetric stretching mode
axial	equatorial				
^{16}O	^{16}O	1201	—	1414	—
^{16}O	^{17}O	1192	9	1401	13
^{17}O	^{16}O	1186	15	1407	7
^{16}O	^{18}O	1183	18	1392	22
^{18}O	^{16}O	1172	29	1401	13
^{17}O	^{18}O	1170	31	1384	30
^{18}O	^{17}O	1163	38	1389	25
^{18}O	^{18}O	1157	44	1378	36

them chemically distinguishable as was possible with cyclic phosphate diesters. The analysis of chiral [^{16}O,^{17}O,^{18}O]sulphate monoesters presented, therefore, a new conceptual problem.

The frequency of an infrared (IR) vibrational mode is markedly affected if isotopic substitution occurs in the functional group responsible. However, the magnitude of the isotope shift is difficult to predict except in the simplest of molecules, but the shift caused by replacing ^{16}O with ^{18}O in a functional group could be up to 40 cm^{-1} (Pinchas & Laulicht 1971). We expected that the generalized anomeric effect (Kirby 1983) of the axial lone pairs of the ring oxygens on the axial exocyclic oxygen in 2,2-dioxo-1,3,2-dioxathianes, would ensure that the frequencies of the symmetric and antisymmetric $> SO_2$ vibrational modes were depen-

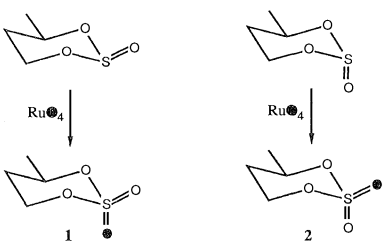
dent on the location of the heavy oxygen isotope. If so, the isotope effect could form the basis of a method for the analysis of chiral [^{16}O,^{17}O,^{18}O]sulphate monoesters.

To explore the influence of oxygen isotopic substitution at the axial and equatorial sites of six-membered cyclic sulphate esters, 2,2-[^{18}O]dioxo-1,3,2-dioxathiane was prepared from 2-oxo-1,3,2-dioxathiane by oxidation with ruthenium [$^{18}O_4$]tetraoxide (Lowe & Salamone 1983), since this should exist as an equimolar mixture of chair conformations (neglecting the equilibrium isotope effect) with ^{18}O in the axial and equatorial sites.



The FTIR spectrum is shown in figure 1*a* (the ^{18}O site is only about 60% enriched). It is evident from this spectrum that the ^{18}O shifts in both the symmetric and antisymmetric stretching modes of the $>SO_2$ group are conformationally dependent. It is also apparent that the isotope shift is greater and the intrinsic linewidth smaller in the symmetric stretching mode; consequently this vibrational mode should be the most useful for analytical purposes. Separate deconvolution of these two spectral regions gave the resolution-enhanced spectrum shown in figure 1*b*.

To assign the conformations responsible for the two symmetric and two antisymmetric $>S[^{16}O,^{18}O]$ absorption bands in 2,2-[^{18}O]dioxo-1,3,2-dioxathiane, and to confirm this observation, the *cis*- and *trans*-cyclic sulphite esters obtained by treating (3*R*)-butane-1,3-diol with thionyl chloride were oxidized with ruthenium [$^{18}O_4$]tetraoxide to give the isotopomeric cyclic [^{18}O]sulphate esters. Since this oxidation is known to proceed with retention of configuration at sulphur (Lowe & Salamone 1983), the *cis*-sulphite must give the cyclic (R_S)-[^{18}O]sulphate **1** and the *trans*-sulphite must give the cyclic (S_S)-[^{18}O]sulphate **2**. As expected, the (R_S)-isotopomer **1** possesses only one symmetric (1172 cm^{-1}) and one antisymmetric (1401 cm^{-1}) $>S[^{16}O,^{18}O]$ stretching vibration and likewise the (S_S)-isotopomer **2** possesses only one symmetric (1183 cm^{-1}) and one antisymmetric (1392 cm^{-1}) $>S[^{16}O,^{18}O]$ stretching vibration, since the conformation with the methyl group equatorial should be preferred. Although these values differ slightly (1 cm^{-1}) from those observed for 2,2-[^{18}O]dioxo-1,3,2-dioxathiane, the assignments shown in figure 1 are unambiguous.



The three stable oxygen isotopes, ^{16}O,^{17}O, and ^{18}O can be arranged to give nine exocyclic isotopomers of (4*R*)-4-methyl-2,2-dioxo-1,3,2-dioxathiane. The ^{17}O^{17}O isotopomer will not appear in the FTIR analysis but the other eight isotopomers will and have been prepared and their FTIR spectra determined. The frequencies of the symmetric and antisymmetric stretching modes for each isotopomer are shown in table 1. As each of the diastereoisotopomeric pairs are distinguishable, IR spectroscopy should provide a means for analysing chiral [^{16}O,^{17}O,^{18}O]sulphate monoesters after stereospecific cyclization to a chirally substituted six-membered cyclic sulphate.

(S_S)- and (R_S)-(1*R*)-3-Hydroxy-1-methylpropyl [^{16}O,^{17}O,^{18}O]sulphates **7** and **8** were prepared as outlined in figure 2*a*. [^{18}O]Thionyl chloride, prepared from sulphur [$^{18}O_2$]dioxide (99 atom% ^{18}O) and phosphorus pentachloride, was used to prepare the *cis*- and *trans*-(4*R*)-4-methyl-2−[^{18}O]oxo-1,3,2-dioxathianes **3** and **4** from (3*R*)-butane-1,3-diol. The separated diastereoisomers were oxidized with ruthenium [$^{17}O_4$]-tetraoxide (prepared *in situ* from ruthenium dioxide, sodium periodate, and [^{17}O]water). Because this oxidation is known to proceed with retention of configuration at sulphur (Lowe & Salamone 1983), the *cis*-[^{18}O]sulphite **3** gives the (2*S*)-compound **5** and the *trans*-[^{18}O]sulphite **4** gives the (2*R*)-compound **6**.

The hydrolytic cleavage of 4-methyl-2,2-dioxo-1,3,2-dioxathiane has been extensively studied, but no conditions had been reported which gave exclusive cleavage of the primary C–O bond (Lichtenberger 1948; Lichtenberger & Durr 1956). Ammonia in methanol, however, gave the desired mode of ring cleavage, the primary amines being isolated virtually quantitatively. The corresponding primary alcohols **7** and **8** were obtained by treatment with nitrous acid in 83% yield.

It was now necessary to develop a stereospecific method for the cyclization of the enantiomeric [^{16}O,^{17}O,^{18}O]sulphate monoester **7** and **8**. Lack of precedent for the formation of cyclic sulphate esters from acyclic sulphate monoesters led to the exploration of several possible reagents. Only two were found, namely trifluoromethanesulphonic anhydride and sulphuryl chloride, the latter giving slightly better yields.

To investigate whether there was any isotope exchange during cyclization, (1*R*)-3-hydroxy-1-methylpropyl[^{18}O]sulphate was prepared and cyclized with sulphuryl chloride. The chemical ionization mass spectrum (NH_3) of the cyclic sulphate obtained revealed a molecular ion at *m/z* 172 only (relative molecular mass, M_r for $C_4H_8SO_3{}^{18}O\cdot NH_4^+$ is 172), suggesting that cyclization had occurred by activating the primary alcohol followed by intramolecular displacement by the sulphate monoester (figure 2*b*). This mode of cyclization was confirmed by the natural abundance ^{13}C NMR spectrum of the cyclic sulphate which showed C-1 to be split into two resonances at δ 71.784 and 71.749 p.p.m., the endocyclic ^{18}O causing an upfield shift of 0.035 p.p.m. as expected (Risley & Van Etten 1979; Vederas 1980; Hansen 1983) and in a 2:1 ratio of intensity after correcting for the ^{18}O enrichment of the sulphate monoester; thus no loss of isotope had occurred. It was now of interest to investigate the FTIR spectrum of the mixture of isotopomeric cyclic sulphate esters. As expected, three absorption bands

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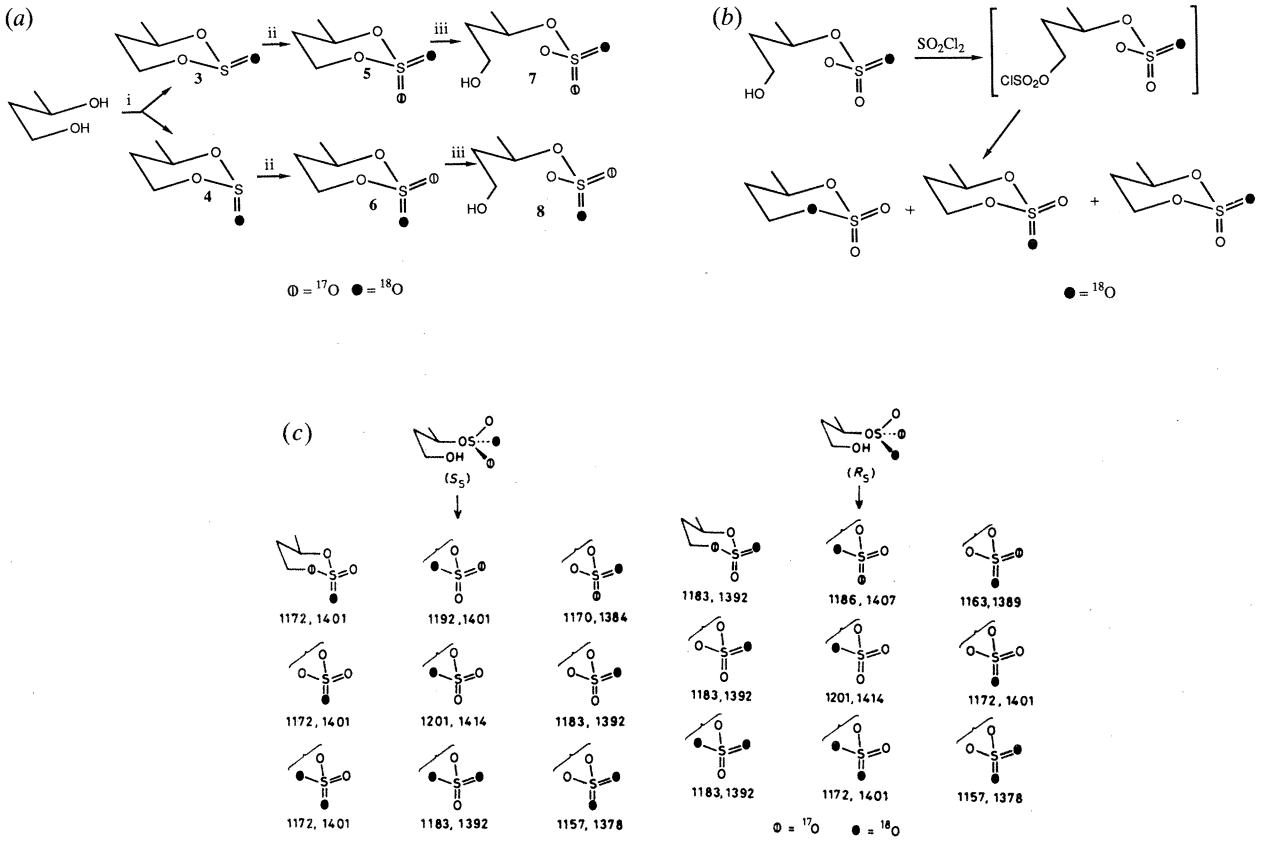

Figure 2*a–e*. (*a*) The synthesis of the (S_S)- and (R_S)-sulphates **7** and **8**. Reagents: i, $S^{18}OCl_2$, C_5H_5N; ii, $Ru^{17}O_4$ (from RuO_2, $NaIO_4$, and $H_2{}^{17}O$; iii, NH_3, MeOH; $NaNO_2$, aq. AcOH. (*b*) The mechanism of cyclization of (1*R*)-3-hydroxy-methylpropyl sulphate. (*c*) The cyclization of the (S_S)- and (R_S)-sulphates **7** and **8** with retention of configuration at sulphur. If the three isotopes were fully enriched only the first three isotopomers of each set would be formed, but in practice the '^{17}O site' contains substantial amounts of ^{16}O and ^{18}O and consequently nine isotopomers should be formed for each chiral [^{16}O,^{17}O,^{18}O]sulphate. The frequency (cm^{-1}) of the symmetric and antisymmetric $>SO_2$ stretching bands for each isotopomer are shown below each formula. (*d*) The synthesis of phenyl [^{16}O,^{17}O,^{18}O]-sulphate **9**. (*e*) The stereochemical course of sulphuryl transfer catalysed by the aryl sulphotransferase from *Aspergillus oryzae*. Although the configuration of the phenyl [^{16}O,^{17}O,^{18}O]sulphate and the *p*-cresyl [^{16}O,^{17}O,^{18}O]sulphate have not yet been determined, both gave 1-O-benzoyl-3(*R*)butane-diol-3-[^{16}O,^{17}O,^{18}O]-sulphate on chemical transfer to 1-O-benzoyl-3(*R*)butane-diol with the same configuration at sulphur. The enzyme catalysed sulphuryl transfer must therefore have proceeded with retention of configuration at sulphur.

were observed in both the symmetric and antisymmetric $>SO_2$ stretching regions (figure 3). For the isotopomer containing ^{18}O in the C–O–S bridge the symmetric and antisymmetric $>SO_2$ absorption bands were at 1201 and 1414 cm^{-1}, respectively, i.e. identical (at 1 cm^{-1} resolution) with those for (4*R*)-4-methyl-2,2-dioxo-1,3,2-dioxathiane (and consequently not resolved from a small amount of unlabelled material). Thus a heavy oxygen isotope in the C–O–S bridge of the cyclic sulphate ester leaves both the symmetric and antisymmetric $>SO_2$ stretching frequencies unperturbed.

As none of the S–O bonds are broken in the cyclization of (1*R*)-3-hydroxy-1-methylpropyl sulphate with sulphuryl chloride the cyclization should proceed stereospecifically for a chiral [^{16}O,^{17}O,^{18}O]sulphate with retention of configuration. In order to confirm this prediction the [(*S*)-^{15}O,^{17}O,^{18}O]-sulphate ester **7** and the [(*R*)-^{16}O,^{17}O,^{18}O]sulphate ester **8** were cyclized with sulphuryl chloride and the FTIR spectra of the isotopomeric mixture of cyclic sulphate esters measured. The spectra of the symmetric and antisymmetric $>SO_2$ stretching frequencies are shown in figure 4.

Figure 2*c* shows the mixture of isotopomeric (4*R*)-4-methyl-2,2-dioxo-1,3,2-dioxathianes that should be formed by cyclizing the (S_S)- and (R_S)-chiral [^{16}O,^{17}O,^{18}O]sulphate esters **7** and **8** with retention of configuration at sulphur by the mechanism outlined in figure 2*b*. If all three isotopes were fully enriched only the three isotopomers shown on the top row of each set would be obtained, but in practice the '^{17}O-site' consists of a substantial amount of ^{16}O and ^{18}O, and therefore nine isotopomeric species should be formed; the ^{18}O site is 99% atom ^{18}O. The symmetric and antisymmetric $>SO_2$ stretching frequencies are shown for each isotopomer.

The spectra shown in figure 4*a*, *b* are easily distinguishable, and therefore provide a method for the stereochemical analysis of chiral [^{16}O,^{17}O,^{18}O]sulphate esters of unknown configuration (Lowe & Parratt 1988).

SYNTHESIS OF PHENYL [^{16}O,^{17}O,^{18}O]SULPHATE

To study both chemical and enzyme catalysed sulphuryl transfer reactions phenyl [^{16}O,^{17}O,^{18}O]-sulphate of known absolute configuration was required. The route developed for the synthesis of phenyl [^{16}O,^{17}O,^{18}O]sulphate is outlined in figure 2*d*. [^{18}O]Thionyl chloride, prepared by the reaction of [$^{18}O_2$]sulphur dioxide with 1,4-bis(trichloromethyl)-benzene in the presence of a catalytic amount of ferric chloride (Hepburn & Lowe 1989), was reacted first with phenol (one equivalent) and then 3β-cholestanol (one equivalent) to give a single crystalline diastereoisomeric [^{18}O]sulphite diester. An X-ray crystal structure analysis of the unlabelled material is in progress which, when complete, should establish the absolute configuration at sulphur. The [^{18}O]sulphite diester was oxidized with ruthenium [^{17}O]tetroxide (generated by isotopic exchange between ruthenium tetroxide and [^{17}O]water). The [^{17}O,^{18}O]sulphate diester on treatment with tetrabutylammonium azide in methylene chloride released phenyl [^{16}O,^{17}O,^{18}O]sulphate. Since none of the sulphur–oxygen bonds are perturbed in this process, and since the oxidation of sulphite diesters to sulphate diesters occurs with retention of configuration at sulphur (Lowe & Salamone 1983) the absolute configuration the phenyl [^{16}O,^{17}O,^{18}O]-sulphate will be established when the configuration at sulphur in the sulphite diester has been determined.

CHEMICAL AND ENZYME CATALYSED SULPHURYL TRANSFER REACTIONS

The tetrabutylammonium salt of the phenyl [^{16}O,^{17}O,^{18}O]sulphate and 1-O-benzoyl-3(*R*)butane-diol were dissolved in carbon tetrachloride and the solution kept at 100 °C for 16 h in a sealed vessel by which time complete transfer had occurred. After debenzoylation of the 1-O-benzoyl-3(*R*)butane-diol-3-[^{16}O,^{17}O,^{18}O]sulphate and extraction of phenol, cyclization was performed on the pyridinium salt with sulphuryl chloride. From the FTIR analysis of the purified isotopomers of (4*R*)-4-methyl-2,2-dioxo-1,3,2-dioxathiane it was clear that the 1-O-benzoyl-3(*R*)-butane-diol-3-[^{16}O,^{17}O,^{18}O]sulphate had the (S_S) configuration. Although the stereochemical course of the chemical transfer will not be known until the

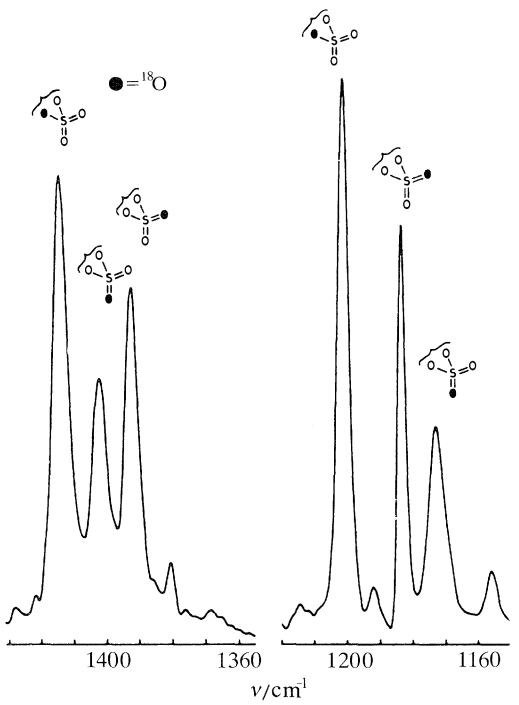

Figure 3. The FTIR spectrum showing the symmetric and antisymmetric $>SO_2$ stretching frequencies of the isotopomeric mixture obtained by cyclizing (1*R*)-3-hydroxyl-1-methylpropyl [^{18}O]sulphate with sulphuryl chloride. The symmetric and antisymmetric SO_2 stretching frequencies at 1201 and 1414 cm^{-1}, respectively coincide with those for unlabelled (4*R*)-4-methyl-2,2-dioxo-1,3,2-dioxathiane. Only partial structures, showing the isotopic arrangement around sulphur are shown.

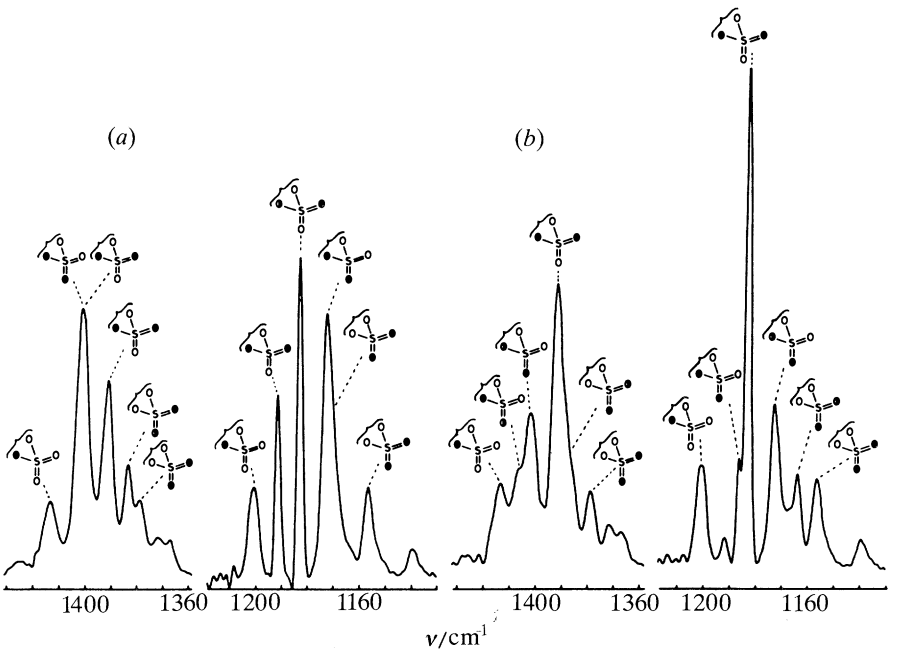

Figure 4. The FTIR spectra showing the symmetric and antisymmetric $>SO_2$ stretching frequencies of the isotopomeric mixture of 4-methyl-2,2-dioxo-1,3,2-dioxathianes obtained by cyclizing with sulphuryl chloride: (*a*) the [(*S*)-^{16}O,^{17}O,^{18}O]sulphate **7** in which the '^{17}O-site' consists of 37.4 atom % ^{16}O, 36.4 atom % ^{17}O, and 26.2 atom % ^{18}O; (*b*) the [(*R*)-^{16}O,^{17}O,^{18}O]sulphate **8** in which the '^{17}O-site' consists of 36.0 atom % ^{16}O, 37.1 atom % ^{17}O, and 26.9 atom % ^{18}O. Only partial structures, showing the isotopic arrangement around sulphur, are shown.

configuration of the phenyl [^{16}O,^{17}O,^{18}O]sulphate has been established this result allows the stereochemical investigation of enzymic reactions to be determined.

Phenyl [^{16}O,^{17}O,^{18}O]sulphate was now incubated with a two molar equivalents of *p*-cresol in the presence of the aryl sulphotransferase from *Aspergillus oryzae* (figure 2*e*). This enzyme was originally considered to be an aryl sulphatase until it was realized that the rate of transfer was greatly increased if a phenolic acceptor was available (Burns & Wynne 1975). Thus in the presence of a phenolic acceptor the sulphatase activity is suppressed. Interestingly, the enzyme was shown not to require adenosine 3′,5′ diphosphate as a co-factor but a kinetic study suggested the involvement of a covalent sulpho-enzyme intermediate, the transfer of the sulphuryl group from donor to acceptor occurring by a ping-pong type mechanism (Burns *et al.* 1977).

The [^{16}O,^{17}O,^{18}O]sulphuryl transfer was allowed to proceed only until 10 % of the phenyl [^{16}O,^{17}O,^{18}O]-sulphate had been depleted, since if the reaction proceeds with inversion of configuration and the product *p*-cresyl [^{16}O,^{17}O,^{18}O]sulphate could act as a donor, partial or even total racemization would occur. The phenyl- and *p*-cresyl [^{16}O,^{17}O,^{18}O]sulphates were separated by HPLC and the configuration of the *p*-cresyl [^{16}O,^{17}O,^{18}O]sulphate analysed by transferring the [^{16}O,^{17}O,^{18}O]sulphate to 1-O-benzoyl-3(*R*)butane-diol in carbon tetrachloride at 100 °C for 16 h as above. After debenzoylation of the 1-O-benzoyl-3(*R*)butane-diol-3-[^{16}O,^{17}O,^{18}O]sulphate followed by cyclization with sulphuryl chloride, FTIR analysis showed that the spectrum was essentially identical with that derived from the phenyl [^{16}O,^{17}O,^{18}O]sulphate. Clearly, the *p*-cresyl [^{16}O,^{17}O,^{18}O]sulphate had the same configuration at sulphur as the phenyl [^{16}O,^{17}O,^{18}O]sulphate and hence the enzymic reaction proceeds with retention of configuration at sulphur. Thus, in this first investigation of the stereochemical course of a sulphotransferase, it is gratifying to find that the kinetic and stereochemical methods are in accord, sulphuryl transfer occurring by a ping pong mechanism with a sulpho-enzyme intermediate on the reaction pathway.

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