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Structure–Activity Studies of Sulfate Transfer: the Hydrolysis and Aminolysis of 3'-Phosphoadenosine 5'-Phosphosulfate (PAPS)

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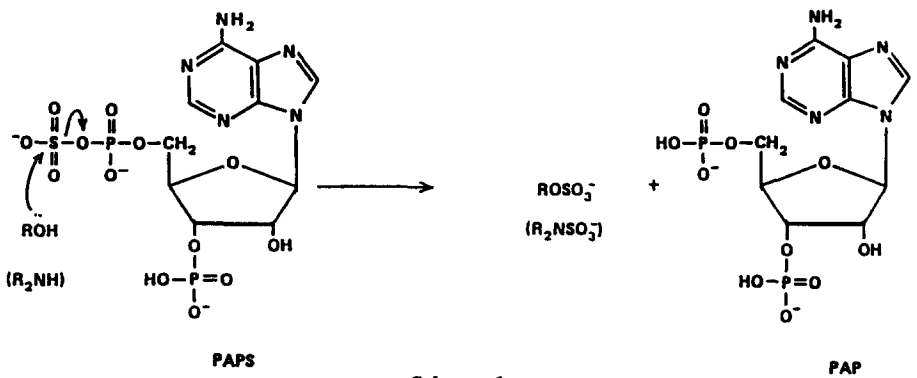
Abstract—The pH–rate profile for the hydrolysis of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) in aqueous solution has been measured. Comparison with other data suggests that hydrolysis occurs by almost complete unimolecular elimination of sulfur trioxide, with weak involvement of a molecule of water in the transition state. The catalytic power (k_{cat}/k_{uncat}) of the sulfotransferases is estimated to be in the order of 10^{10}–10^{12}. Amines—exemplified by morpholine—react spontaneously with PAPS in water at 39 °C by attack at both sulfuryl and (5')phosphoryl groups in a ratio of 2:3. The mechanism of activation of the coenzyme, PAPS, by the sulfotransferases that catalyse *N*-sulfation must involve suppression of its native *N*-phosphorylating reactivity and specific enhancement of its *N*-sulfating reactivity. Studies of the aminolysis of the coenzyme in aprotic solvent–water mixtures suggest how this might be accomplished.

Introduction

Biological sulfation is accomplished by enzymes that use as coenzymes the sulfated derivatives of nucleotides. The major form of 'active sulfate', identified in 1956 by Robbins and Lipmann,[2] is 3'-phosphoadenosine 5'-phosphosulfate (PAPS). This coenzyme is employed in the sulfation of many classes of biomolecule, ranging from proteins, lipids, and polysaccharides (heparin and chondroitin are examples) to bile acids, steroidal hormones, and perhaps ascorbic acid. It is also employed in the conjugation of foreign compounds[3]—important historically since biological sulfation was first recognised as a phenomenon in 1875 by Baumann[4] who isolated phenyl sulfate ($PhO{\bullet}SO_3H$) from the urine of a patient dosed with phenol. 'Sulfate conjugates' of foreign compounds are of topical interest too, since much of what we know of the enzymology of biological sulfation has derived from studies with purified hepatic sulfotransferases that have foreign compounds as substrates.[3]

These PAPS-dependent processes effect, in the main, either *O*-sulfation or *N*-sulfation of a substrate. In all cases, whether the substrate be a large or small (bio)molecule, the sites (e.g. hydroxyl or amino groups) of sulfation are, by definition, nucleophilic groups. Since *O*- and *N*-sulfation are mechanistically congeneric, the prototype biological sulfation reaction can be taken as the sulfotransferase-mediated sulfation of an alcohol, ROH, by PAPS. At its simplest, it can be viewed as a sulfotransferase-mediated nucleophilic displacement at S with expulsion of 3'-phosphoadenosine 5'-phosphate (PAP) (Scheme 1).

Biological sulfation is one of several important transferase-catalysed processes, and the one closest to biological phosphorylation. Mechanistically we can



Scheme 1

group these two closely-related biological 'acylation' reactions with the three major biological alkylation processes, methylation, prenylation, and glycosylation. Each effects the acylation or alkylation of a nucleophilic grouping in a biomolecule by means of a coenzyme in which the group to be transferred is attached to a good leaving group. The coenzymes involved are adenosine 5'-triphosphate (ATP)/phosphotransferases, *S*-adenosylmethionine (SAM)/methyltransferases, dimethylallyl pyrophosphate (DMAPP)/prenyltransferases and uridine-5-diphospho-α-D-glucose (UDPG)/glucosyltransferases.

The catalytic power of these transferases has long been of interest to mechanistic bio-organic chemists, and more than twenty years ago that of the phosphotransferases (kinases) came under close scrutiny. By elucidating the weak spontaneous (non-enzymic) phosphorylating activity of adenosine 5'-triphosphate (ATP) at pH 7, it was possible to calculate from known enzymic rates that the catalytic power (k_{cat}/k_{uncat}) of the phosphotransferases was in the order of 10^{10}–10^{12} (see Benkovic[5]). Subsequently, Schowen and Coward and co-workers[6] determined the spontaneous methylating reactivity of *S*-adenosylmethionine (SAM) and estimated the catalytic power of the methyltransferases to be in the order of 10^{16}. No attempt has been made to evaluate the catalytic power of the prenyltransferases, although Tidd's determination of the spontaneous alkylating reactivity of DMAPP and related compounds[7] provides the necessary information. Recently we determined the spontaneous glucosylating reactivity of UDPG and estimated[8] the catalytic power of the glycosyl transferases to be in the order of 10^{10}. However, there have been, to our knowledge, no studies of the measurement of the spontaneous sulfating reactivity of PAPS which would yield similar data relevant to the magnitude of the catalytic role of the sulfotransferases. We have now accomplished this task, and report rates of hydrolysis of PAPS in the pH range 1–7. We have also studied the reactivity of two *N*-nucleophiles (a secondary and a tertiary amine) towards the PAPS dianion, in order to compare their reactivity with *O*-nucleophiles (alcohols, phenols) and to throw light on the mechanism by which sulfotransferases catalyse biological *N*-sulfation. Our results include measurements in both water and in mixtures of dimethyl sulfoxide–water, and show that amines can attack PAPS at both sulfur and phosphorus.

Results

Hydrolysis of PAPS

In order to follow the hydrolysis of PAPS by quantitative HPLC, using spectrophotometric detection of its adenosine chromophore, a paired-ion system capable of resolving PAPS from its only adenosine-containing hydrolysis product, PAP was developed (Fig. 1). Neither the 3'- nor the 5'-phosphate group of PAP was found to be labile under the conditions studied, as expected,[9] and PAP was the only product observed.

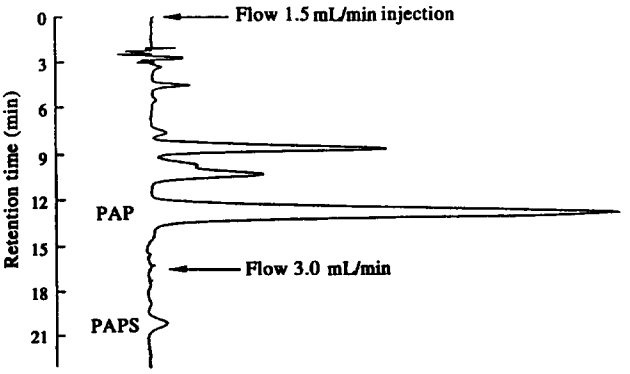


Figure 1. HPLC trace of a mixture of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) and 3'-phosphoadenosine-5'-phosphate (PAP).

From the results of pilot experiments and for comparison with related reactions, a temperature of 39 °C was chosen for the determination of the pH–rate profile for hydrolysis. The kinetic data is summarised in Table 1 and the results plotted in Figure 2. Values of k_{obs} may be calculated from equation 1, where k_{H+} is the second order rate constant associated with hydronium ion-catalysed hydrolysis of the monoanion species,[§] k_M and k_D are the first order rate constants for the hydrolysis of the monoanion and dianion, respectively, and a_{H+} is the activity of the hydrogen ion as measured by a glass electrode.

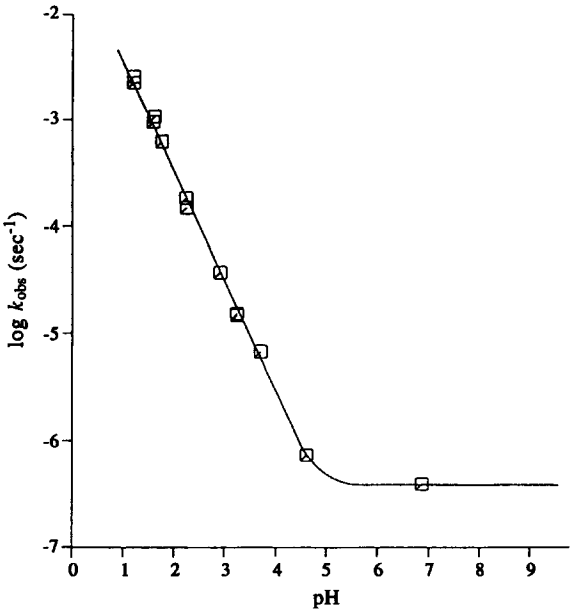


Figure 2. The pH–rate profile of the hydrolysis of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) at 39 °C and ionic strength 0.1M. The curve is calculated from the rate constants listed

[§]In discussions of the ionisation of the phosphosulfate group, the ionisation of the 3'-phosphate has been ignored.

$$k_{obs} = k_{H+}[a_{H+}^2/(a_{H+} + K_a)] + k_M[a_{H+}/(a_{H+} + K_a)] + k_D[K_a/(a_{H+} + K_a)] \quad (1)$$

Table 1. Observed rate constants for the hydrolysis of PAPS in aqueous soloution (39 °C, $\mu = 0.1$)

Buffer	Molarity (M)	pH	$10^7 k_{obs}$ (s^{-1})
HCl	0.10	1.21	$2.3 \pm 0.10 \times 10^4$
HCl	0.10	1.21	$2.6 \pm 0.05 \times 10^4$
HCl	0.05	1.59	$10.0 \pm 0.20 \times 10^3$
HCl	0.05	1.62	$10.6 \pm 0.10 \times 10^3$
HCl	0.03	1.76	$6.2 \pm 0.20 \times 10^3$
HCl	0.01	2.24	$1.8 \pm 0.02 \times 10^3$
HCl	0.009	2.25	$1.5 \pm 0.03 \times 10^3$
HCl	0.003	2.92	$3.6 \pm 0.06 \times 10^2$
HCl	0.001	3.24	$1.4 \pm 0.07 \times 10^2$
HCl	0.001	3.24	$1.5 \pm 0.04 \times 10^2$
Formate	0.10	3.70	$6.8 \pm 0.25 \times 10$
Acetate	0.10	4.60	7.3 ± 0.30
Phosphate	0.05	6.86	3.9 ± 0.36

The acid dissociation constant K_a was calculated assuming a pK_a for the first protonation of the phosphosulfate moiety of 2.4, the pK_a of phenyl-phosphosulfate.[10] The values of the derived rate constants are shown in Table 2. The equation can be written as the rate law:

$$d[PAP]/dt = k_{H+M}[\text{monoanion}][H^+] + k_M[\text{monoanion}] + k_D[\text{dianion}] \quad (2)$$

which implies that both the monoanion and the dianion are labile.

Table 2. Derived rate constants for the hydrolysis of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) at 39 °C and ionic strength 0.1 M

k_{H+}	$4.0 \pm 0.05 \times 10^{-2}$ mol^{-1} s^{-1}
k_M	$1.0 \pm 0.06 \times 10^{-4}$ s^{-1}
k_D	$3.9 \pm 0.36 \times 10^{-7}$ s^{-1}

Aminolysis of PAPS

The rate of aminolysis of PAPS by a secondary and by a tertiary amine was followed by quantitative HPLC, using spectrophotometric detection. The derived second order rate constants for aminolysis of PAPS by DABCO and morpholine at 39 °C in water are shown in Table 3.

Table 3. Second order rate constants for the aminolysis of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) in water at 39 °C and ionic strength 0.02 M

Amine	Rate constant (k_2)
morpholine	$1.49 \pm 0.10 \times 10^{-4}$ mol^{-1} s^{-1}
DABCO	$2.14 \pm 0.02 \times 10^{-4}$ mol^{-1} s^{-1}

Since 3'-phosphoadenosine 5'-(*N*-morpholinyl)phosphoramidate (PAMP) and PAP were both found to be stable under the conditions of hydrolysis, the amounts of PAMP and PAP formed in the aminolysis of PAPS by morpholine (Fig. 3) give a direct measure of the amounts of P–O and S–O fission, respectively. At 39 °C the values found (after correcting for the PAP content of the sample of PAPS used) were 61% P–O fission and 39% S–O fission.

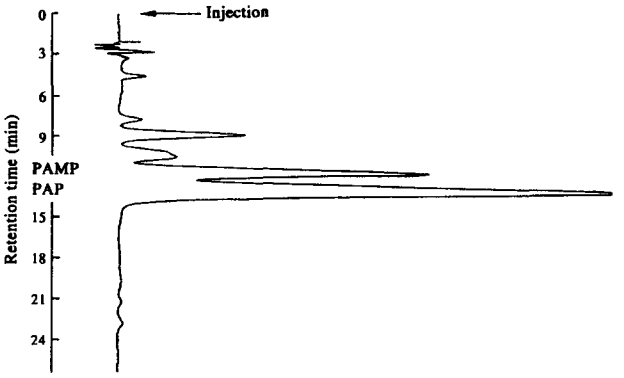


Figure 3. HPLC trace of a mixture of 3'-phosphoadenosine 5'-(*N*-morpholino)phosphoramidate (PAMP) and 3'-phosphoadenosine 5'-phosphate (PAP).

Hydrolysis and aminolysis of PAPS in DMSO–water mixtures

As the rates of hydrolysis and aminolysis (morpholine) of PAPS in high concentrations of dimethyl sulfoxide (90 and 95% v/v) were very low, it was possible only to estimate rate constants under these conditions. These data are shown in Table 4.

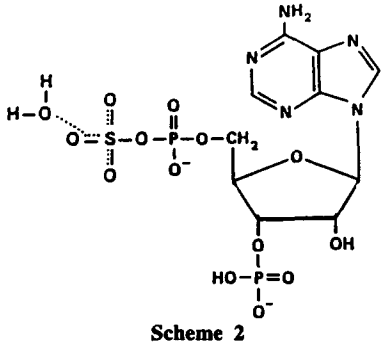
Table 4. Estimated rate constants for the hydrolysis and aminolysis (morpholine) of 3'phosphoadenosine 5'-phosphosulfate (PAPS) in mixtures of dimethyl sulfoxide (DMSO) and water at 39 °C and ionic strength 0.02 M

Volume % DMSO	Hydrolysis Rate constant (k_1)	Aminolysis Rate constant (k_2)
0	2.30×10^{-7} s^{-1}	1.49×10^{-4} mol^{-1} s^{-1}
90	$< 7.7 \times 10^{-8}$ s^{-1}	ca. 4.0×10^{-7} mol^{-1} s^{-1}
95	$< 7.0 \times 10^{-8}$ s^{-1}	$<1.5 \times 10^{-7}$ $mol^{-1}s^{-1}$

Discussion

The interpretation of the observed rate profile for PAPS is much simplified by a consideration of the conclusions drawn by Benkovic and Hevey[10] in their comprehensive study in 1970 of the hydrolysis at 55 °C of phenyl phosphosulfate (PPS). This compound, which was studied as an easily accessible model for PAPS, has a phenyl group in place of the 3'-phosphoadenosyl residue of PAPS, and it was reasoned by Benkovic and Hevey[10] that, because of its 'insulation' from the reacting centre by the phosphate group, this should not unduly alter the reactivity of the sulfuryl group towards nucleophilic reagents. Our results confirm that view, on two counts. First, the shape of the profile for PAPS at 39 °C (Fig. 1) is very similar to that[10] of PPS at 55 °C, with each showing a region (pH 6–8) where the rate is independent of pH, and a region (pH 2–5) where the rate increases with decreasing pH, the slope of the line being −1. Second, by using rate data for PPS determined[10] at 37 °C and our own data for PAPS at 39 °C, we estimate the reactivity of the monoanion of PPS to be only about half that of PAPS.

^{18}O-Labelling studies by Benkovic and Hevey[10] confirmed that PPS is hydrolysed by S–O fission, and we can safely assume that PAPS hydrolyses similarly. We presume that the mechanism of the hydrolysis of the dianion (and of the monoanion) of PAPS is similar to that proposed[10] for PPS, i.e. almost complete unimolecular elimination of sulfur trioxide with weak involvement of a molecule of water in the transition state (Scheme 2).



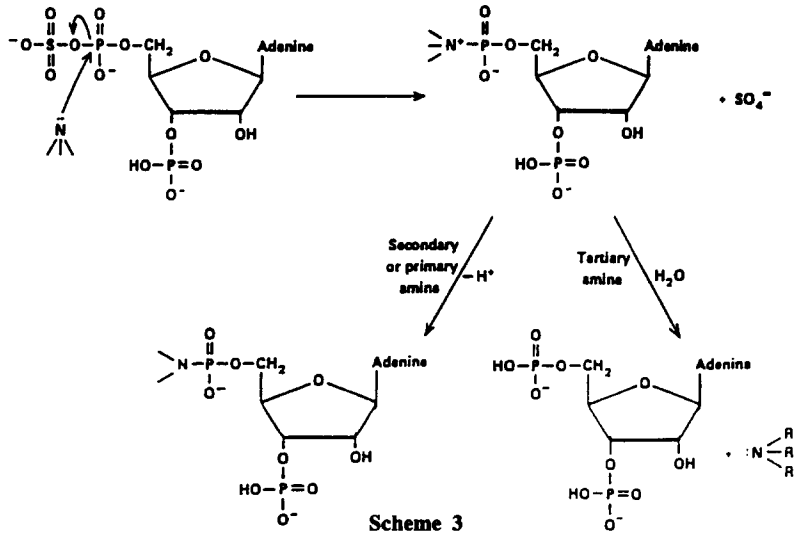
Scheme 2

The amines chosen for the aminolysis study were 1,4-diazobicyclo[2.2.2]octane (DABCO) and morpholine, a tertiary and a secondary amine respectively. In earlier work, Tagaki and Eiki[11] showed that the aminolysis of phenyl phosphosulfate (PPS) by morpholine involves attack at both phosphorus and sulfur, in a ratio of about 4:1. This is in contrast with the behaviour towards hydrolysis and methanolysis which they,[11] and Benkovic and Hevey,[10] showed to involve virtually exclusive S–O fission. In view of our finding that the reactivity of PAPS towards water is very similar to that of PPS, we expected that the aminolysis of PAPS would also involve both P–O (Scheme 3) and S–O fission (Scheme 4). The aminolysis products formed by P–O fission of PPS by morpholine were inorganic sulfate and phenyl *N*-morpholinylphosphoramidate,[11] which is stable under the conditions used. The expected organic product from the analogous reaction between PAPS and morpholine is therefore PAMP (Scheme 3). On the other hand, the product of attack at PAPS phosphorus by DABCO, a tertiary amine, is of a type known to break down rapidly to the free amine and PAP (Scheme 3). The adenosine-containing product of attack on sulfur by either amine is PAP (Scheme 4).

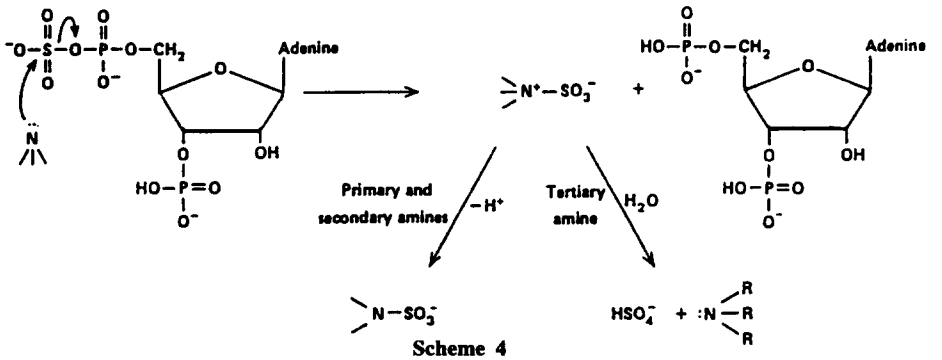
Our results show that attack by morpholine at the phosphoryl group is about 1.5 times faster than at the sulfuryl group. Comparable data for PPS show that attack by morpholine is four times faster at phosphorus than at sulfur.[11] We explain this difference in reactivity as follows. AMP and PPS are similar leaving groups, so a given nucleophile should attack PAPS and PPS *sulfur* at similar rates. The increase from 1.5:1 (PAPS) to 4:1 (PPS) for P–O versus S–O cleavage by morpholine is thus primarily a measure of the lower reactivity at the PAPS *phosphorus*. The magnitude of the effect, a factor of about two to three, is due to the different 'spectator' groups, with a pK_a difference of about six (PhO versus RCH_2O). This weak effect of a 'spectator' group is similar to that observed in the reaction of nucleophiles with phosphorus diesters. For example, when the pK_a difference of the 'spectator' groups in a pair of phosphate diesters—bi(2,4-dinitrophenyl) phosphate[12a] and methyl 2,4-dinitrophenyl phosphate[12b]—was about twelve (2,4-dinitrophenoxy versus MeO) and the leaving group was 2,4-dinitrophenate, the reactivity ratio towards 4-aminopyridine (pK_a 9.11) (statistically corrected by a half) was 5.5–6:1.[12] Since we would expect a similar rate ratio for morpholine (pK_a 8.36), this would point to a rate difference of about three for spectator groups with a pK_a difference of six. The observed phosphosulfate *phosphorus* reactivity ratio of 2.6:1 for PPS versus PAPS is thus in line with the data for P diesters.

The rate constants for the aminolysis of PAPS (Table 3) are similar in magnitude to those found for the aminolysis of PPS by Tagaki and Eiki.[11] These workers showed that the rate of aminolysis of PPS by secondary amines has only a small dependence on the pK_a of the amine, the Brönsted coefficient (β_{nuc}) being 0.15. This is characteristic of a mechanism involving only a small degree of bond formation prior to the transition state. Dividing by two to allow for the two amino groups present in DABCO, it can be seen that the rates of aminolysis of PAPS by DABCO and morpholine are very similar. This is consistent with a mechanism for the aminolysis of PAPS involving only a small degree of bond formation to sulfur in the transition state (Scheme 5). Attack at phosphorus is analogous to the reaction of a diester with a good leaving group (sulfate in this case), and involves a tighter transition state binding to the nucleophile (Scheme 6).[12a]

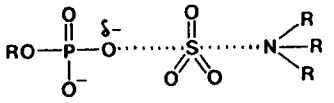
The large decreases in the rates of both hydrolysis and aminolysis of PAPS in high concentrations of the aprotic solvent, dimethyl sulfoxide (DMSO) (Table 4) are consistent with those formulations of the transition



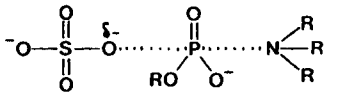
Scheme 3



Scheme 4



Scheme 5



Scheme 6

states, and of that of the related transition state of the hydrolysis reaction (Scheme 2). In the ground state each of the two negative charges is discretely associated with the phosphate and sulfate groups, but in the transition states the leaving group, whether it be sulfate or phosphate, possesses two almost fully formed negative charges. Such a localisation of negative charge means that aprotic solvent-stabilisation by ion–dipole interactions is much less than that in the ground state where the charges are separated. This is in marked contrast with results for *p*-nitrophenyl phosphate[13] and sulfate,[14] where charge is more delocalised in the transition state and hydrolysis is accelerated in DMSO. These results suggest that the sulfotransferases responsible for *N*-sulfation could suppress the phosphorylating activity and enhance the sulfating activity of PAPS by providing at the active site separate hydrophobic and hydrophilic 'compartments' for the (5') phosphoryl group and the sulfuryl group of PAPS.

Our results show that the dianion of PAPS (the major species at physiological pH) is attacked very slowly by water at pH 7, having a half-life of more than 20 days. The calculated second order rate constant for the attack of water upon PAPS is 7×10^{-9} M^{-1} s^{-1}. Expressing this in a way suitable for comparison with an enzymic reaction, and converting to units familiar to enzymologists, we can say that the spontaneous (non-enzymic) sulfation of water by PAPS occurs at a rate of 4.2×10^{-10} mM^{-1} min^{-1}. In the presence of a purified hepatic hydroxysteroid sulfotransferase, for example, the enzymic sulfation of a substrate sterol (ROH) by PAPS occurs[3a] at the rate of about 2×10^{3} mM^{-1} min^{-1}. If the spontaneous rate of sulfation of an alcohol (ROH) by PAPS is similar in magnitude to that of water, this gives a catalytic factor for the hydroxysteroid sulfotransferase of about 10^{12}. Other sulfotransferases, for example some of the phenol sulfotransferases,[3b] are less powerful as catalysts by factors of 10-100. This gives a range of catalytic power for the sulfotransferases of 10^{10}–10^{12}. This range is identical to that estimated for the phosphotransferases,[5] and similar to the range of 10^{11}–10^{13} recently re-estimated[15] for the glycosyltransferases.

Experimental

Materials

3'-Phosphoadenosine 5'-phosphosulfate (PAPS), 3'-phosphoadenosine 5'-phosphate (PAP), adenosine 5'-

phosphosulfate (APS), adenosine 5'-phosphate (5AMP), and adenosine 3'-phosphate (3AMP) were obtained from Sigma Chemical Co. Ammonium dihydrogen phosphate and tetrabutylammonium hydroxide (TBAH) were obtained from BDH Chemical Co., and HPLC-grade methanol from Rathburn Ltd. Morpholine, 1,4-diazobicyclo[2.2.2]octane (DABCO), and dimethyl sulfoxide (DMSO) were obtained from Aldrich Chemical Co. DABCO was recrystallised twice from toluene, and morpholine was fractionally distilled twice from sodium and used immediately. DMSO was fractionally distilled under house vacuum twice from calcium hydride and used immediately.

Chromatographic methods

Apparatus. A pair of HPLC pumps with control box (LDC, Constametric II) in combination with a mixer (Altex) and a pressureless injection system (Waters Assoc., Model U6K) were used for solvent delivery and application. The analytical column (250 × 5 mm) (Hichrom) was packed with LiChrosorb RP-18 (10 μm: Merck). A Co:Pell ODS guard column (Hichrom) was mounted between the pumps and the injection valve. The absorbance of the nucleotides was monitored at 259 nm using a Cecil UV Detector (Model CE212) and peak areas and retention times were calculated with a CCM HPLC Controller (LDC). The CCM was also used to program the pumps. The injection volume was 25 μL.

Solvents for paired-ion chromatography. The mobile phase was 0.0475 M tetrabutylammonium hydroxide, 0.0475 M ammonium dihydrogen orthophosphate (pH 8.0) in aqueous 25% methanol. The eluant was monitored at λ = 259 nm. For optimal analysis of the products of aminolysis using morpholine as reactant, the concentration of tetrabutylammonium hydroxide was increased to 0.06 M.

Kinetic studies

The kinetics of a PAPS hydrolysis reaction were followed by quantitative HPLC either by periodically measuring the decrease in peak area of PAPS or the increase in peak area of PAP (the only detectable product). All reactions were studied at 39.0 ± 0.1 °C and were initiated by adding a known volume of pre-heated buffer to a known weight of PAPS. In all cases the PAPS dissolved immediately. Buffers employed for hydrolysis of PAPS were HCl (pH < 3.5), phosphate (0.1 M), acetate (0.1 M), and formate (0.1 M) with ionic strengths of 0.1 made up with sodium chloride; a buffer of 0.02 M TBAH was used with an ionic strength of 0.02. No corrections of the rate data for buffer catalysis were necessary, since representative checks for buffer catalysis at pH 3.7 (formate) and pH 6.9 (phosphate) showed there to be none. For aminolysis reactions and the reactions in high concentrations of DMSO (also studied at 39.0 ± 0.1 °C) the ionic strength was kept at 0.02 using TBAH. All pH values were measured using a Russel glass electrode and a Corning pH meter. For reactions whose fast rate made it impossible for them to be followed accurately by direct injection, 50 μL aliquots were removed and quenched in 100 μL 0.5 M carbonate buffer. Pseudo-first-order rate constants were calculated from data acquired over at least four half-lives. Rates of aminolysis were obtained from the slopes of pseudo-first-order rate constants versus the concentration of amine.

Product ratios

An estimate of the amount of S–O and P–O bond cleavage occurring during aminolysis of PAPS by morpholine was obtained by calculating, respectively, the amounts formed (using peak areas) of 3'-phosphoadenosine 5'-phosphate (PAP) and of 3'-phosphoadenosine 5'-(*N*-morpholinyl)-phosphoramidate (PAMP). (Allowance was made for a small amount of PAP present in the starting material.)

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