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Studies on Sulphatases

20. ENZYMIC CLEAVAGE OF ARYL HYDROGEN SULPHATES IN THE PRESENCE OF $H_2^{18}O^*$

By B. SPENCER

Division of Cancer Research, Washington University Medical School, St Louis, Missouri, and Department of Biochemistry, University of Wales, Cardiff†

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Two types of arylsulphatase have been recognized (Dodgson & Spencer, 1957*a*), the distinction between them being based on their relative substrate affinities and their behaviour towards various inhibitors. Type I arylsulphatases have an appreciable activity towards monopotassium *p*-nitrophenyl sulphate (NPS) and monopotassium *p*-acetylphenyl sulphate (APS) but are less active towards dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS). Type II enzymes have the reverse specificity, being invariably highly active towards NCS but only feebly active towards NPS and APS. The arylsulphatases are widely distributed in nature but so far only vertebrates are known to possess both type I and type II enzymes simultaneously.

* Part 19: Dodgson & Wynn (1958).

† Present address.

The premises on which speculations about the function of these two types of arylsulphatase may be based are few. The fundamental issue, which has not yet been decided, is whether the enzymes exert a purely hydrolytic role or whether this is only a reaction *in vitro* and that *in vivo* they actually act as transferases. Two transferring activities can be envisaged involving the transfer of either the phenol or the sulphate group. In order to decide which of these reactions may be possible, the position of cleavage of arylsulphates by arylsulphatases has been studied by hydrolysis of the substrates in $H_2^{18}O$ followed by isolation of the liberated sulphate and assay of its ^{18}O content. Cleavage of the C-O bond (1) will yield inorganic sulphate containing no ^{18}O , whereas if the O-S bond is split (2) the ^{18}O content of the liberated sulphate will be one-quarter of the atoms per cent

^{18}O of the aqueous medium. Knowledge of the site of cleavage will allow deductions to be made concerning the possible mechanism of action of the enzymes.

EXPERIMENTAL

Materials

Arylsulphates. Monopotassium *p*-acetylphenyl and *p*-nitrophenyl sulphates were prepared by the method of Burkhardt & Lapworth (1926). Dipotassium 2-hydroxy-5-nitrophenyl sulphate was freed from impurities by recrystallization of the monopotassium salt (Dodgson & Spencer, 1956). H_2^{18}O of 1.4 atoms % excess was purchased from the Stuart Oxygen Company, San Francisco, California, U.S.A.

Enzymes. Arylsulphatase *C* was prepared from rat-liver microsomes treated with Lissapol. To the microsomes obtained by the method of Schneider (1948) from 16 g. of rat liver was added 1 g. of Lissapol-N (Imperial Chemical Industries Ltd.), the volume made up to 25 ml. with ice-cold water and the whole adjusted to pH 9.8 with NaOH. After standing for half an hour at 0° the pH was lowered to 7.5 with a drop of conc. HCl and the solution centrifuged at G_{max} 105 500 *g* for 1 hr. at 0° . The supernatant was poured off and acetone added to it to give a final concentration of 80% (v/v). After standing for 15 min. at 0° to flocculate, the precipitated proteins were collected by vacuum filtration, washed well with ice-cold acetone and dried *in vacuo*. When required for ^{18}O studies a portion of the powder (250 mg.) was washed ($\times 3$) with 20 ml. of water by homogenizing with a glass homogenizer and centrifuging at G_{max} 3500 *g* for 15 min. The resulting precipitate was washed with 5 ml. of H_2^{18}O on the centrifuge and the final precipitate suspended in 7 ml. of H_2^{18}O to give a final volume of 10 ml. The arylsulphatase *C* so prepared was still slightly contaminated with the arylsulphatases *A* and *B* of the microsomal fraction.

Acetone-dried cells of *Alcaligenes metalcaligenes* (2.9 g.) were treated according to the directions of Dodgson, Melville, Spencer & Williams (1954) to give preparation *B*, except that the final powder was produced by freeze-drying rather than by precipitation with acetone. The freeze-dried powder was dissolved in 3.5 ml. of H_2^{18}O immediately before use.

Human arylsulphatase *A* was the freeze-dried preparation 1 of Baum, Dodgson & Spencer (1958). In the experiments with ^{18}O the dried material (30 mg.) was dissolved directly in the reaction mixture.

Methods

Briefly, the method adopted was to treat the aryl hydrogen sulphates with the hydrolytic agent in the presence of as high a concentration of H_2^{18}O as possible. Samples (5 μl .) of the reaction mixture were removed at

intervals and the extent of hydrolysis was assessed by spectroscopic measurement of the liberated phenols. When sufficient hydrolysis had taken place to ensure an adequate sample of BaSO_4 for measurements of ^{18}O , the reaction was stopped and the liberated sulphate precipitated as BaSO_4 . Various washing procedures were employed in an attempt to avoid adsorption of contaminants on the precipitated BaSO_4 .

In the acid hydrolysis, 80 mg. of NPS and APS or 110 mg. of NCS was dissolved in 4 ml. of H_2^{18}O contained in a 15 ml. tapered centrifuge tube, and 80 μl . of 10 *N*-HCl was added. A portion (0.3 ml.) of the mixture was removed for estimation of the ^{18}O content and the remainder heated on a boiling-water bath for 20 min. Complete hydrolysis occurred under these conditions. After cooling, the reaction mixture was extracted with 5 ml. of ether ($\times 3$) to remove the liberated phenol. After the removal of the residual ether by a current of clean air, the liberated sulphate was precipitated by the addition of 3 ml. of 5% (w/v) BaCl_2 . The precipitate was sedimented on the centrifuge and washed ($\times 4$) with 7.5 ml. of water, followed by 10 ml. of ethanol ($\times 2$) and 10 ml. of ether. The washed BaSO_4 was dried over P_2O_5 *in vacuo* for 2 days. A control, containing 55 mg. of K_2SO_4 instead of the aryl hydrogen sulphate, was incubated and treated identically.

Alkaline hydrolysis was carried out by adding 320 mg. of NaOH and 160 mg. of NPS (or 55 mg. of K_2SO_4 in the control experiment) to 4 ml. of H_2^{18}O contained in a 15 ml. tapered centrifuge tube. After removal of 0.4 ml. of the reaction mixture for estimation of ^{18}O the tube was heated in a boiling-water bath for 2 hr., which resulted in 90% hydrolysis. After cooling, the solution was made acid with 1 ml. of 10 *N*-HCl, extracted with ether and the BaSO_4 isolated as in the acid-hydrolysis experiments.

The isolation of BaSO_4 after enzymic hydrolysis required the precipitation of protein with trichloroacetic acid before addition of BaCl_2 and the subsequent treatment of the BaSO_4 precipitate with NaOH in an attempt to remove adsorbed protein. To the reaction mixture after incubation was added the required volume of 30% (w/v) trichloroacetic acid, to give a final concentration of 5%, due allowance being made for the buffer concentration and pH of the reaction mixture. The precipitated protein was removed by centrifuging and the supernatant shaken with 5 ml. of ether, a slight scum forming at the ether-water interface. The aqueous layer was removed with a pipette (the scum being left behind) and extracted with 5 ml. of ether ($\times 2$). After removal of residual ether, 3 ml. of 5% (w/v) BaCl_2 was added, the precipitate collected by centrifuging and washed with 7.5 ml. of water ($\times 4$). The BaSO_4 was then suspended in 5 ml. of 0.5 *M*-NaOH, allowed to stand for 10 min., centrifuged and the supernatant removed. The precipitate was washed successively with 5 ml. of 0.5 *N*-HCl, 5 ml. of water ($\times 3$), 10 ml. of ethanol ($\times 2$) and 10 ml. of ether. The final precipitate was dried *in vacuo* over P_2O_5 for 2 days.

The compositions of the reaction mixtures in the enzyme experiments differed with each enzyme and as far as possible were designed to reproduce the optimum pH and substrate concentration for the particular enzyme and substrate concerned. For arylsulphatase *C* the reaction mixture contained 3 ml. of enzyme suspension in H_2^{18}O containing 0.4 g. of 2-amino-2-hydroxymethylpropane-1:3-diol and 5.7 mg. of NaF, the whole being adjusted to

pH 8 with a drop or two of acetic acid. A portion (0.3 ml.) of the mixture was removed for measurement of ^{18}O and the remainder incubated at 37° . After 12 hr., 30% hydrolysis of the NPS had occurred and 31% of NCS. The incubation was continued for a total of 23 hr., during which time 48% of the NPS and 45% of the NCS had been hydrolysed. Sodium fluoride was added to suppress arylsulphatase *A* and *B* activity (Dodgson, Spencer & Wynn, 1956).

The arylsulphatase of *Alcaligenes metalcaligenes* (1 ml.; see above) was added to 4 ml. of 0.02M- K_2HPO_4 dissolved in H_2^{18}O and adjusted to pH 8.75 with a few crystals of KH_2PO_4 . To this mixture was added 13 mg. of NPS (0.01M final concentration), 87 mg. of NCS (0.05M) or 55 mg. of K_2SO_4 . Samples (0.3 ml.) were removed for analysis of ^{18}O and the remainder was incubated at 37° for 18 hr. Further 30 mg. additions of NPS were made at 1, 2 and 15 hr. and a single addition of 70 mg. of NCS at 2 hr. This procedure was adopted to avoid the complete inhibition of the enzyme by excess of substrate which has been observed previously (Dodgson *et al.* 1954). The NPS was hydrolysed to the extent of 93%, and for NCS the figure was 59%.

Human arylsulphatase *A* (30 mg.; see above) and 130 mg. of NPS (0.125M) were added to 4 ml. of 0.5M-sodium acetate in H_2^{18}O and adjusted with a drop or two of acetic acid to pH 6. With NCS (174 mg., 0.125M) the pH was adjusted to 4.9. Controls containing 55 mg. of K_2SO_4 instead of the substrate were run at both pH values. After removal of samples (0.3 ml.) for analysis of ^{18}O the reaction mixtures were incubated at 37° for 24 hr., during which time 18% hydrolysis of NPS and 49% hydrolysis of NCS had occurred.

Analyses. The extent of hydrolysis of APS, NPS and NCS was assessed by adding samples (5 $\mu\text{l.}$) of the reaction mixture to the appropriate volumes of 0.5N-NaOH and measuring the liberated *p*-hydroxyacetophenone at 323 $\text{m}\mu$ (ϵ_{323} 20 500), *p*-nitrophenol at 400 $\text{m}\mu$ (ϵ_{400} 18 200) and 4-nitrocatechol at 515 $\text{m}\mu$ (ϵ_{515} 11 200). Isotope ratios were determined on a mass spectrometer of the Nier type by Dr Mildred Cohn. The water of the reaction mixture was analysed for ^{18}O in the form of CO_2 after equilibration with CO_2 for 2 days. The oxygen of the samples of BaSO_4 (less

than 20 mg. was required) was also measured as CO_2 after being converted into a mixture of CO_2 and CO in the manner described by Hoerring (1952).

RESULTS

Some slight exchange of ^{18}O between water and sulphate occurred in the control experiments, in which K_2SO_4 was incubated with H_2^{18}O under conditions identical with those used in the hydrolysis of the aryl hydrogen sulphates (Table 1). The exchange of oxygen between water and sulphate is known to take place at a slow rate in neutral solution and at a faster rate in acid and alkali (Hoerring, 1952; Datta, Day & Ingold, 1937), and the presence of a maximum of 0.018 atom % excess ^{18}O in the BaSO_4 isolated from the enzyme control experiments cannot therefore be regarded as showing any marked enzymic catalysis of oxygen transfer similar to that observed between phosphate and water in the presence of lobster-muscle strips (Stein & Koshland, 1952).

The ^{18}O contents of the samples of BaSO_4 isolated after acid and enzymic hydrolysis of the aryl hydrogen sulphates (Table 1) were within 85.3–98.4% of the theoretical values required for the splitting of the bond between the oxygen and sulphur atoms. The discrepancies between the experimental and theoretical values can probably be attributed to the adsorption of oxygen-containing organic compounds on to the precipitated BaSO_4 , resulting in a dilution of the ^{18}O obtained after conversion of the oxygen of the sample into CO and CO_2 . This contamination of the sample was kept to a minimum by the use of stringent washing precautions designed particularly to remove protein and the liberated phenol. Some contamination was undoubtedly due to adsorption by BaSO_4 of the unhydrolysed substrate and, indeed, the

Table 1. *Hydrolysis of aryl hydrogen sulphates in H_2^{18}O*

Type of hydrolysis	Agent	Substrate	Concn. of ^{18}O (atom % excess) in BaSO_4			
			Found	Calc. for		Sulphate control
				$\text{ArO};\text{SO}_3\text{H}^*$	$\text{Ar};\text{OSO}_3\text{H}$	
Acid	0.2 N-HCl	NPS	0.315	0.330	0	0.014
		NCS	0.316	0.330	0	0.014
		APS	0.325	0.330	0	0.014
Alkaline	2 N-NaOH	NPS	0.210	0.333	0	0.014
Enzymic, Type I arylsulphatase	Rat-liver arylsulphatase <i>C</i>	NPS	0.224	0.244	0	0.002
		NCS	0.208	0.244	0	0.002
	Arylsulphatase of <i>Alcaligenes metal-</i> <i>caligenes</i>	NPS	0.267	0.333	0	0.010
		NCS	0.303	0.333	0	0.010
Enzymic, Type II arylsulphatase	Human liver arylsulphatase <i>A</i>	NPS	0.290	0.322	0	0.018
		NCS	0.300	0.322	0	0.010

* The calculated value is equal to one-quarter of atom % excess in the water of the reaction mixture.

samples of BaSO₄ isolated after enzymic hydrolysis of NCS were coloured yellow, the colour of the unhydrolysed substrate. The BaSO₄ obtained after acid hydrolysis of NCS was not coloured, but in this case complete hydrolysis of the sulphate ester was achieved and the experimental ¹⁸O value was 95.8 % of the theoretical. The extremely low value for the ¹⁸O content of the BaSO₄ isolated after hydrolysis of NPS with the arylsulphatase of *Alcaligenes metalcaligenes* (80.2 % of the theoretical value for O-S cleavage) was due to accidental contamination of the BaSO₄ with dirt after the breakage of the centrifuge tube. Unfortunately, this experiment could not be repeated owing to lack of enzyme material, but since the enzymic reaction can be expected to cleave only one type of bond the high ¹⁸O content of the barium sulphate in this case, although below the theoretical value, is certainly indicative of O-S cleavage.

In the barium sulphate isolated after alkaline hydrolysis of NPS the ¹⁸O content was only 63 % of the theoretical amount required for O-S cleavage. So low a figure cannot be explained by adsorption of impurities on the precipitated BaSO₄, particularly since there could be no protein contamination. Moreover, another portion of the isolated BaSO₄ did not lose weight after heating at 650° for 2 hr.

DISCUSSION

From Table 1 it is clear that acid hydrolysis of aryl hydrogen sulphates (APS, NPS, NCS) involves the cleavage of the O-S bond and presumably takes place through attack by the electrophilic hydroxonium ion. These experimental findings confirm the original suggestion of Burkhardt, Ford & Singleton (1936), based on the fact that aryl hydrogen sulphates do not show phenylating properties similar to the alkylating properties of the alkyl sulphates, where cleavage of the C-O bond is indicated. The further suggestion of these workers that the alkaline hydrolysis of *p*-nitrophenyl hydrogen sulphate is mediated through C-O bond cleavage is not wholly supported by the studies with ¹⁸O. In actual fact, only one-third of the *p*-nitrophenol liberated from potassium *p*-nitrophenyl hydrogen sulphate could be derived from C-O bond splitting. Adequate controls showed that this result could not be explained by transfer of the ¹⁸O from H₂¹⁸O to the liberated sulphate or vice versa, but the possibility that some transfer of ¹⁸O occurred between water and the NPS in alkaline solution before hydrolysis could not be ruled out. Further investigations on the mechanism of the alkaline hydrolysis of aryl hydrogen sulphates and particularly of the serum- and amino acid-catalysed alkaline hydrolyses (Dodgson & Spencer, 1957*b*) of NPS would obviously be of interest.

The point of cleavage of oxygen-bridge compounds in enzymic reactions may be deduced according to a generalization derived by Koshland & Stein (1954), if it is assumed that the reactions proceed by displacement mechanisms. This generalization has been stated thus: 'If the enzyme shows high specificity for the group R of the ROQ substrate and low specificity for the group Q, cleavage of the R-O bond occurs in the enzymatic reaction.' Although the arylsulphatases have a high specificity towards the aryl group, in that they are not known to hydrolyse sulphate esters other than those of phenols, considerable latitude is observed in the substituents allowed in the benzene ring. Even the 'phenolic' sulphate ester of kojic acid is attacked (Soda, Katura & Yoda, 1940). On the other hand, the specificity of these enzymes towards the sulphate group appears to be absolute. On these grounds it could be predicted from Koshland's generalization that arylsulphatases would break the bond between the oxygen and sulphur atoms. Such, indeed, appears to be the case, for the results in Table 1 show that Type I and Type II arylsulphatases each catalyse the cleavage of the O-S bond of the aryl hydrogen sulphates, which are the specific substrates of both Type I and Type II enzymes. These results are in accord with Koshland's generalization and may be taken to indicate that the mechanism of arylsulphatase action involves a displacement reaction. However, since the arylsulphatase reactions may be specific rather than belonging to a group to which a generalization may be applied, neither supposition is unequivocal.

A hypothetical reaction mechanism was proposed by Dodgson, Spencer & Williams (1956) for the hydrolysis of aryl hydrogen sulphates by the Type I arylsulphatase of *Alcaligenes metalcaligenes*, based on the observed changes in Michaelis constant with pH and the effect of substituents in the aromatic ring of phenyl hydrogen sulphate on the *K_m* and *V_{max}* of the enzyme. The scheme shown (Scheme 1) involved a displacement reaction in which the sulphate group on the enzyme was displaced under attack by the electrophilic hydroxonium ion. The increased rate of hydrolysis of substrate resulting from the introduction of electrophilic substituents into the substrate was thought

Scheme 1

to be due to the greater stability of the product $\text{Ar}\cdot\ddot{\text{O}}$, whereas the other product $\text{SO}_2\cdot\text{O}^-$ was presumed to be stabilized by the combination with the enzyme. The major assumption in the proposed mechanism was that fission occurred between the oxygen and sulphur atoms, and the present work with ^{18}O has now shown this to be true. Furthermore the supposition derived from Koshland's generalization, i.e. that a displacement reaction is involved, is also in full accord with this scheme. It would appear that both Type I and Type II arylsulphatase functions by displacement mechanisms and that the fundamental difference between the two types of enzyme lies in the nature of their substrate-binding groups (cf. Dodgson & Wynn, 1958) rather than in the different mechanisms of breakdown of the enzyme-substrate complexes.

By analogy with the enzymes which split phosphate esters (cf. Axelrod, 1956) and by consideration of the mechanism of the aryl hydrogen sulphate hydrolysis it is possible to state that if the arylsulphatases act as transferases they will catalyse the transfer of the sulphate group rather than that of the phenol. The transfer of sulphate from NPS to 3':5'-diphosphoadenosine to give adenosine-3'-phosphate-5'-phosphosulphate (active sulphate) is known to take place under the influence of an enzyme obtained from mammalian livers, but there are several reasons for believing that none of the mammalian arylsulphatases already described can catalyse this reaction (Baum & Dodgson, 1958). Suzuki, Takahashi & Egami (1957) have observed the transfer of sulphate from NPS to carbohydrate materials under the influence of acetone-dried mucous glands of *Charonia lampas*, and there is some evidence that arylsulphatases may be involved. These findings, although by no means unequivocal, are the first hint that arylsulphatases may actually be involved in sulphate transfer. Attempts to demonstrate transfer of sulphate from NPS to a number of simple alcohols, sugars, choline and tyrosine in the presence of purified human arylsulphatase *A* or *B* have so far been unsuccessful (K. S. Dodgson & F. A. Rose, unpublished work).

SUMMARY

1. The hydrolysis of aryl hydrogen sulphates by acid, alkali and enzymes was carried out in H_2^{18}O ,

followed by isolation of the liberated sulphate and determination of its ^{18}O activity.

2. Hydrolysis of monopotassium *p*-nitrophenyl sulphate and dipotassium 2-hydroxy-5-nitrophenyl sulphate by human-liver arylsulphatase *A*, rat-liver arylsulphatase *C*, the arylsulphatase of *Alcaligenes metalcaligenes* and by acid resulted in cleavage of the O-S bonds. Only two-thirds of the theoretical amount of ^{18}O for O-S cleavage was found in the barium sulphate isolated after alkaline hydrolysis of monopotassium *p*-nitrophenyl sulphate.

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