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L-ASCORBIC ACID 2-SULPHATE

A SUBSTRATE FOR MAMMALIAN ARYLSULPHATASES

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Summary

Ascorbic acid 2-sulphate has a stability in acid comparable to that of phenyl sulphate and is rather more acid-labile than simple carbohydrate sulphates.

At its optimum pH of 4.8 sulphatase A(aryl-sulphate sulphohydrolase EC 3.1.6.1.) hydrolyses ascorbic acid sulphate with a specific activity of 90 μmol/mg per min (150 μmol/mg per min with nitrocatechol sulphate at pH 5.6). At pH 4.8 the kinetics are non-Michaelis. At pH 5.6 Michaelis kinetics are obeyed and K_m is 21 mM ascorbic acid 2-sulphate. K_2SO_4 is a competitive inhibitor with a K_i of 0.2 and 0.6 mM at pH 4.8 and 5.6, respectively. Sulphatase A is converted into a substrate-modified form during its hydrolysis of ascorbic acid sulphate.

Sulphatase B also hydrolyses ascorbic acid 2-sulphate. At pH 4.8 and in the presence of 0.15 M NaCl the specific activity is 0.92 μmol/mg per min (90 μmol/mg per min for nitrocatechol sulphate at pH 5.6). In the absence of NaCl the activity is greatly decreased. K_m is 8 mM. K_2SO_4 is a competitive inhibitor with a K_i of 0.1 mM.

Ascorbic acid is not hydrolysed at a detectable rate by the arylsulphatases of the mollusc *Dicathais orbita* or of *Aerobacter aerogenes*.

Introduction

Ascorbic acid 2-sulphate (originally thought to be the 3-sulphate) was first found in the undeveloped cysts of the brine shrimp *Artemia salina* [1,2] and more recently in a number of rat tissues [3] and in human urine [4]. The mild oxidation of ascorbic acid 2-sulphate in vitro is accompanied by transfer of the sulphate group to a suitable acceptor [5—7] and it has been postulated [1]

that a similar reaction might occur in vivo. Verlangieri and Mumma [8] showed that administration of ascorbic acid 2-[^{35}S]sulphate to rats led to the appearance of about 7% of the ^{35}S as cholesteryl sulphate in the faeces but the mechanism of the reaction was not elucidated. It could have been an oxidative transfer analogous to that in vitro, or it could have occurred indirectly.

Although itself a rather acid-stable compound [9], it has been suggested [2] that ascorbic acid 2-sulphate can form a compound of high 'sulphate potential', comparable to that of 4-nitrophenyl sulphate, during oxidation. It therefore seemed possible that it might be a substrate for arylsulphatases, especially for sulphatase A which, as well as showing arylsulphatase and cerebroside sulphatase activities [10], can hydrolyse galactose 3-sulphate [11]. The present communication describes an investigation of the action of some arylsulphatases on ascorbic acid 2-sulphate.

Recently Hatanaka et al. [12] have described the hydrolysis of ascorbic acid 2- and 6-sulphates by extracts of the liver (hepatopancreas or digestive gland) of the marine gastropod *Charonia lampas*. Such extracts are known to show many types of sulphatase activity.

Experimental

L-Ascorbic acid 2-sulphate

This was prepared by the method of Quadri et al. [13] except that the barium salt was crystallised twice from boiling water (about 100 ml per 40 g salt) before crystallising from 50% methanol (about 100 ml per 0.4 g salt). Found: C, 16.87; H, 2.43. $C_6H_6BaO_9S \cdot 2H_2O$ requires C, 16.86; H, 2.36. $\epsilon_{254\,nm}$ 18 500 at pH 7.5; $\epsilon_{233\,nm}$ 12 000 at pH 1.

The barium salt was converted to the potassium salt by passage through a column of Dowex 50 X8, K^+ form, and the concentration of the resulting solution determined spectrophotometrically.

Acid hydrolysis

The acid hydrolysis of ascorbic acid 2-sulphate (potassium salt) was followed spectrophotometrically at 233 nm in a Gilford spectrophotometer with the cell compartment maintained at the required temperature. The change in absorbance was relatively small ($\epsilon_{233\,nm}$ 12 000 and 8500 for ascorbic acid 2-sulphate and ascorbic acid, respectively) but was adequate for the present purpose. As ascorbic acid is destroyed in hot acid A_∞ was calculated from the initial concentration of the sulphate. Apparent first-order velocity constants, k', were calculated by standard methods.

Enzymes

Sulphatases A and B were prepared from ox liver [14] and ox brain [15], respectively. Highly purified samples of the arylsulphatases of *Dicathais orbita* (Mollusca: Gastropoda) and of *Aerobacter aerogenes* were gifts from Dr D. Yellowlees and Dr R.G. Nicholls, respectively.

Measurement of enzyme activity

This was done in a pH-stat by titration with 0.015 M NaOH at 37°C. The

exact experimental conditions are specified below but in all cases the volume of the reaction mixture was 10 ml and 0.25 mM EDTA was present. Unless otherwise stated the substrate concentration was 25 mM ascorbic acid 2-sulphate (potassium salt). With sulphatase A the velocity at 1 min after starting the reaction (v_1) was computed by fitting a rectangular hyperbola to the data in the previously described [16] modification of the method of Stinshoff [17]. With sulphatase B the velocities were obtained directly from the slopes of the linear recordings. Rates were corrected for the incomplete ionisation of ascorbic acid (pK 4.1 [2]) in the pH range studied. Kinetic parameters were computed by the method of Wilkinson [18].

Substrate-modified sulphatase A was isolated and assayed with nitrocatechol sulphate as previously described [19].

Results

Acid hydrolysis

The results are shown in Fig. 1. There was a linear relationship between log k' and H_0, the Hammett acidity function: the slopes of the lines were 0.79, 0.84 and 0.86 at 40, 60 and 80°C, respectively. The activation energy for the hydrolysis, calculated from the data in Fig. 1, was 95 800 and 97 800 J/mol at 3.69 M and 4.92 M HCl, respectively: this is of the same order (23 kcal/mol) as values for the hydrolysis of aryl sulphates [20].

In 0.25 M HCl at 80°C, k' was $1.0 \cdot 10^{-3} s^{-1}$: assuming the activation energy to be 96 800 J/mole gives a k' of $6.0 \cdot 10^{-3}$ s^{-1} at 100°C, corresponding to a $t_{1/2}$ of 1.9 min.

Sulphatase A

In preliminary experiments it was noted that sulphatase A hydrolysed ascorbic acid 2-sulphate but the results were less concordant than expected and the reaction sometimes ceased within a few min. As 0.5 mM ascorbic acid rapidly inactivates sulphatase A [21], especially in the presence of Cu^{2+}, 0.25

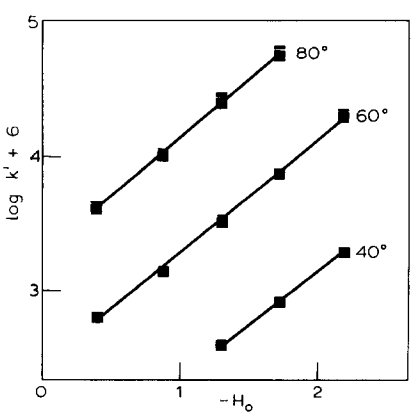

Fig. 1. The relationship between log k', the apparent first-order rate constant for the reaction, and H_0, the Hammett acidity function, for the hydrolysis of ascorbic acid 2-sulphate in 0.1—6 M HCl at the temperatures indicated on the figure.

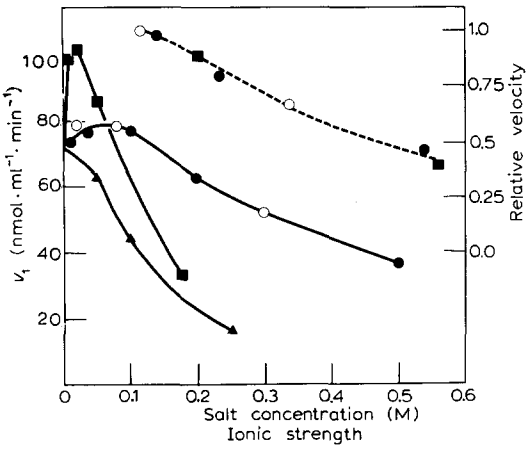

Fig. 2. The effect of salts on the hydrolysis of 12.5 mM ascorbic acid 2-sulphate by sulphatase A (1 μg/ml) at pH 4.8 in the presence of 0.25 mM EDTA. ●, NaCl; ○, KCl; ■, $MnCl_2$; ▲, KNO_3. The dotted line relates relative enzyme activity to the total ionic strength (salt + substrate) of the reaction mixture.

mM EDTA was included in the reaction mixture. This prevented the rapid inactivation of the enzyme and improved the reproducibility to that obtained [16] with nitrocatechol sulphate as substrate: 5 replicate assays gave a v_1 (mean ± S.D.) of 842 ± 24.5 nmol/min.

The pH optimum for the hydrolysis of ascorbic acid 2-sulphate by sulphatase A was 4.7—4.8. The specific activity, determined in 25 mM ascorbic acid sulphate, was 93 and 38 μmol/mg per min at pH 4.8 and 5.6, respectively.

The effect of some ions on the hydrolysis of ascorbic acid 2-sulphate by sulphatase A is shown in Fig. 2. Chlorides initially activate and then inhibit, the effect being particularly apparent with $MnCl_2$. The inhibition appears to be due to increasing ionic strength because the effects of $MnCl_2$ and of NaCl or KCl are similar when compared on this basis. The strong inhibition by KNO_3 (Fig. 2) was surprising because there was no similar effect with nitrocatechol sulphate as substrate [10].

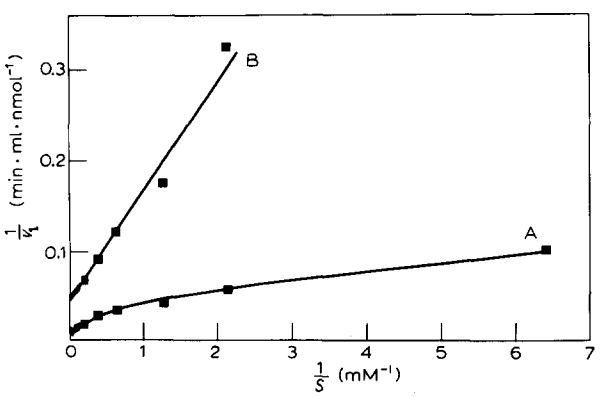

Fig. 3. Reciprocal plots of the effect of varying concentrations of ascorbic acid 2-sulphate on the rate of its hydrolysis (v_1) by sulphatase A in 0.25 mM EDTA. (A) pH 4.8, enzyme concn 1.1 μg/ml. (B) pH 5.6, enzyme concn 2.6 μg/ml. Note that for clarity this curve is displaced 0.04 unit upwards on the ordinate.

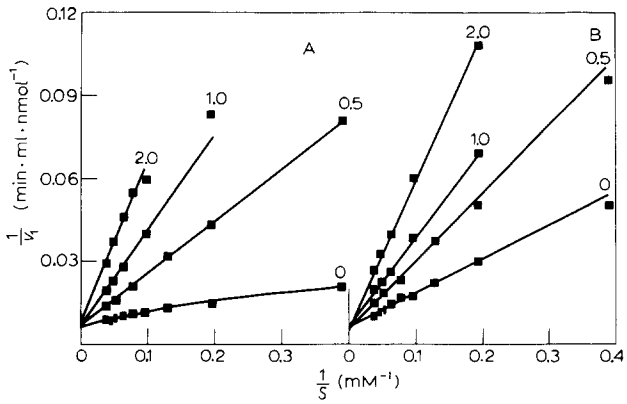

Fig. 4. The effect of K_2SO_4 on the hydrolysis of ascorbic acid 2-sulphate by sulphatase. A. Conditions as in Fig. 3; concentrations (mM) of K_2SO_4 indicated on the figure. (A) pH 4.8, enzyme concentration 1.1 μg/ml. (B) pH 5.6, enzyme concentration 2.2 μg/ml.

The optimum substrate concentration was about 30 mM and above this the velocity decreased, perhaps because of the rapidly increasing ionic strength due to the substrate which is a doubly charged anion in this pH range [2]. As simple salts themselves influence the enzyme (Fig. 2), control of the ionic strength was not attempted but the concentration of the substrate was normally kept below 25 mM (I = 0.075).

The effects of varying substrate concentration on v_1 are shown in Fig. 3: although the reciprocal plot was linear at pH 5.6 it was not at pH 4.8. At pH 5.6 K_m was 21.5 ± 2.2 mM ascorbic acid 2-sulphate. At both pH values the reaction was competitively inhibited by K_2SO_4 (Fig. 4) and replots of the slopes of the reciprocal plots gave values of 0.6 and 0.2 mM K_2SO_4 for K_i at pH 5.6 and 4.8, respectively. Because of the non-Michaelis kinetics the significance of the latter value is not clear but at high substrate concentrations the non-linearity is not obvious (Fig. 4) and the apparent K_m is 7 mM ascorbic acid 2-sulphate at pH 4.8.

Even in the presence of EDTA sulphatase A was slowly inactivated during the hydrolysis of ascorbic acid 2-sulphate. The $t_{½}$ for the inactivation was about 15 and 60 min at pH 4.8 and 5.6, respectively, and the modified enzyme was not activated, but further inhibited, by K_2SO_4. The enzyme isolated [19] after reaction for 1 h with ascorbic acid 2-sulphate at pH 4.8 contained about 60% of a substrate-modified form: this hydrolysed nitrocatechol sulphate only after the addition of K_2SO_4 and was indistinguishable from the substrate-inactivated enzyme obtained after incubation with nitrocatechol sulphate [19].

Sulphatase B

Sulphatase B hydrolysed ascorbic acid 2-sulphate at a relatively low rate. The specific activity, at pH 4.8 in 0.15 M NaCl, with 25 mM ascorbic acid sulphate was 0.92 μmol/mg per min. In the absence of NaCl the hydrolysis was much slower, by a factor of 10 or more, as previously noted for the hydrolysis of substrates other than nitrocatechol sulphate by this enzyme [22].

The pH optimum was close to 4.8 and at that pH Michaelis kinetics were

obeyed: K_m was 8.13 ± 0.59 mM ascorbic acid 2-sulphate. The reaction was competitively inhibited by K_2SO_4 with a K_i of 0.1 mM.

Other arylsulphatases

The arylsulphatase of *D. orbita* at a concentration of 50 μg/ml did not hydrolyse ascorbic acid 2-sulphate at a detectable rate between pH 4.8 and 6.0. Likewise the arylsulphatase of *A. aerogenes* at a concentration of 38 μg/ml had no detectable activity towards ascorbic acid 2-sulphate between pH 4.8 and 7.2.

As a rate of 0.5 μmol/min would have been readily detectable, the specific activities of these enzymes with ascorbic acid 2-sulphate must be less than 0.1 μmol/mg per min, respectively.

Discussion

There is a considerable variation in the values of ϵ reported for ascorbic acid 2-sulphate: these are summarised in Table I. The reason for this variation is not obvious, the more so as there is no comparable variation in the values of λ_{max} or in the ratio $\epsilon_{254nm}/\epsilon_{233nm}$

Ascorbic acid 2-sulphate is, despite statements to the contrary [9], relatively acid-labile and the data in Fig. 1 show it to be hydrolysed by HCl at a rate comparable to phenyl sulphate [23]. In 0.25 M HCl at 100°C it is hydrolysed more rapidly than simple carbohydrate sulphates: the $t_{½}$ for ascorbic acid 2-sulphate is about 2 min while those for the most labile type of carbohydrate sulphate (with an equatorial hydroxyl group esterified) fall between 6 and 25 min [24]. The data in Fig. 1 are consistent with the acid-catalysed hydrolysis of ascorbic acid 2-sulphate being an A-1 reaction, as are the hydrolyses of alkyl sulphates [25], aryl sulphates [20,23] and the sulphate esters of some heterocycles [26].

Some pertinent data for the hydrolysis of ascorbic acid 2-sulphate by arylsulphatases is summarised in Table II.

TABLE I

SPECTRAL DATA REPORTED FOR DIFFERENT PREPARATIONS OF L-ASCORBIC ACID 2-SULPHATE

Ref.	Neutral solution			Acid solution			Ratio $\epsilon_{neutral}/\epsilon_{acid}$
	λ_{max}	$\epsilon_{max} \times 10^{-3}$	pH	λ_{max}	$\epsilon_{max} \times 10^{-3}$	pH	
1	254	35.0*	8.6	230	22.5*	1	1.55
9	254	29.4	7.0	233	21.9	2	1.34
2	254	17		232	11		1.54
3	254	16.0†	7.0	233	12.8†	2.0	1.25
13	255	21.7	7.2	233	14.1	2.1	1.54
12	255	16	7	232	11	2	1.54
Present work	254	18.5	7.5	231	12	1	1.53

* Subsequently reported (ref. 2) to be an error.

† These values were used in ref. 3 without reference to those previously given in ref. 9.

TABLE II

KINETIC DATA FOR THE HYDROLYSIS OF ASCORBIC ACID 2-SULPHATE AND NITROCATECHOL SULPHATE BY SULPHATASES A AND B

Values for V are given in moles substrate hydrolysed per mol enzyme per min at 37°C. The values marked with an asterisk are apparent because of non-Michaelis kinetics (see text).

	Nitrocatechol sulphate			Ascorbic acid sulphate	
	pH	K_m (mM)	V	K_m (mM)	V
Sulphatase A	5.6	0.4	16000	22	7500
	4.8			7*	14500*
Sulphatase B	5.6	1.9	7100		
	4.8			8.1	75

With sulphatase A, the plot of $1/v_1$ against $1/S$ is non-linear at the optimum pH of 4.8 but is linear at pH 5.6 (Fig. 3). At pH 4.8 and at a concentration of about 1 μg/ml sulphatase A exists as a mixture of polymeric forms [27] so that non-Michaelis kinetics would be expected: at pH 5.6, on the other hand, the monomer will be dominant and show simple kinetics. The hydrolysis of ascorbic acid 2-sulphate by sulphatase A is competitively inhibited by K_2SO_4, both at pH 4.8 and 5.6: this is similar to the inhibition of the hydrolysis of nitrocatechol sulphate [10] but not of cerebroside sulphate which is noncompetitively inhibited [10,28].

During its reaction with ascorbic acid 2-sulphate, sulphatase A is converted into a substrate-modified form which, when isolated, is indistinguishable from that formed by reaction with nitrocatechol sulphate [19]. The modification is much slower with ascorbic acid sulphate than with nitrocatechol sulphate and while the latter is favoured by increasing pH [19], the former is favoured by decreasing pH. Again, the rate of hydrolysis of ascorbic acid 2-sulphate by a mixture of substrate-modified and native enzyme is not increased by K_2SO_4: this is contrary to the results with nitrocatechol sulphate and 4-nitrophenyl sulphate [19], but similar to those with 4-methylumbelliferone sulphate (unpublished observations).

Sulphatase B also hydrolyses ascorbic acid 2-sulphate, but much less effectively than sulphatase A (Table II). This reaction is also competitively inhibited by K_2SO_4, an unexpected finding because the hydrolysis of nitrocatechol sulphate by sulphatase B is noncompetitively inhibited [29].

The fact that ascorbic acid 2-sulphate can be hydrolysed by at least two mammalian arylsulphatases further complicates the interpretation of the results of Verlangieri and Mumma [8]. Although direct oxidative transfer of sulphate from ascorbic acid 2-sulphate to cholesterol obviously cannot be excluded, neither can more indirect mechanisms, including hydrolysis to SO_4^{2-} followed by reutilisation of the latter as probably occurs with, for example, 4-nitrophenyl sulphate in the rat [30]. It also throws further doubt on the validity of using these arylsulphatases as analytical tools because it is clear that their specificity is even wider than previously envisaged.

The other arylsulphatases investigated, those of *D. orbita* and of *A. aerogenes*, do not hydrolyse ascorbic acid 2-sulphate at a measurable rate.

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