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Kinetic and Inhibitor Studies with Arylsulfhydrolases A and B from Avian and Mammalian Sources*

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Arylsulfhydrolases A and B from chicken and from bovine liver have been isolated and their reactions with a range of synthetic arylsulfates examined using kinetic methods. Some differences of Michaelis–Menten parameters were observed in comparing the A with the B forms from the two sources at the level of individual substrates. At that level also, interspecies comparisons of A forms and B forms similarly showed differences. However, for none of the four enzymes examined was there consistent correlations of kinetic values with electronic, hydrophobicity, or steric properties of the substrates. The bovine A enzyme displayed the well-documented "anomalous" kinetic behavior at high substrate concentrations; at low concentrations conventional hydrolysis of *p*-nitrocatechol sulfate occurred, except that there was evidence with this substrate and others of product inhibition. The avian A enzyme reacted normally over all substrate concentrations examined, but again product inhibition occurred. The mammalian but not the avian B enzyme was also clearly subject to product inhibition.

INTRODUCTION

The arylsulfatases (arylsulfate sulfhydrolase EC 3.1.6.1) catalyze the hydrolysis of arylsulfates which are not, however, the natural substrates, these latter being, for example, cerebroside and steroid sulfates (*1*, *2*). The arylsulfates nevertheless provide a family of readily synthesized (*3*) substrates useful in examining some aspects of the reactivities of the arylsulfatases. We have lately been engaged in a study encompassing enzymes of both type I and II (*4*) which are distinguished in part by pHs of maximal activity, and are isolated from such sources as bacteria (*Aerobacter aerogenes*) and snails (*Helix pomatia*) (*5*, *6*).

Two arylsulfatases have recently been reported as occurring in chicken liver (*7*) and have been, by analogy with mammalian systems, classified as A and B forms. Further, the A form was described (*7*) as behaving anomalously when the time course of the hydrolysis of *p*-nitrocatechol (*p*-NCS) was followed, another apparent parallelism with the well-known behavior of the mammalian A arylsulfatase (*2*). It was of interest, then, to examine the two sulfhydrolases from chicken liver more extensively in order to determine their relationship with each other and to the mammalian systems.

We here report a study of the kinetics of hydrolysis of a series of synthetic arylsulfates using the enzymes isolated from chicken liver, which were separated

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by affinity chromatographic techniques. The A and B forms of arylsulfatase differ in their behavior on Blue Sepharose CL-6B affinity packing, and (contrast Ref. (*7*)) the A form migrates more slowly to the negative pole of a polyacrylamide disc gel than does the B. Inhibition studies with Ag^+ and Cl^- ions similarly differentiated the two enzymes. Also, the A, unlike the B sulfhydrolase, displayed measurable inhibition in the presence of both SO_4^{2-} and *p*-nitrocatechol, products of the hydrolysis of *p*-NCS for which irregular kinetic behavior was reported (*7*).

The investigation reported here also includes work with enzymes of a mammalian source, bovine liver, so facilitating a comparison of the modes of action of arylsulfatases from a range of organisms. Using analytical methods previously described (*4, 8*), we have confirmed that at high (5 m*M*) *p*-nitrocatechol sulfate concentrations the mammalian A enzyme behaves in an anomalous fashion, which has been attributed to substrate inhibition (*9–11*). At lower concentrations of that substrate the anomaly was no longer evident, though indications of product inhibition which has been reported previously (*4*) were found. The mammalian arylsulfatases examined were not observed to be markedly different from each other in their reactions with the range of arylsulfates studied.

EXPERIMENTAL

Reagents. Unless otherwise noted all reagents were of analytical grade and the water was distilled and deionized.

The arylsulfates employed in the kinetic measurements were those used in a previously reported study (*6*) where synthetic methods and analytical data are described.

Kinetic measurements. These were made spectrophotometrically on Beckman Model 26 and Unicam SP-800 instruments in thermostatted (25°C) cell compartments as has been described (*6, 8*). Michaelis–Menten parameters were estimated from double-reciprocal plots composed of eight substrate concentrations. Pseudo-first-order rate constants k_ψ were independently measured under subsaturating conditions.

Enzyme isolation. Two different methods were employed for the enzymes of chicken liver. (a) The arylsulfatase B was isolated by the procedure originally described by Fedecka-Bruner and Croisille (*7*) and used in some of the measurements reported here. That procedure, however, did not produce the A sulfhydrolase with sufficient activity for extensive kinetic studies, and a second isolation was developed.

(b) Fresh chicken liver (900 g) was defatted, chopped, then macerated in a Waring blender with 1.4 liters of 0.5 *M* acetate buffer, pH 5.3, containing 0.1% Triton X-100, at room temperature. The filtrate from the passage through cheesecloth was centrifuged at 10,000*g* at 4°C for an hour and the supernatant dialyzed versus 0.1 *M* acetate, pH 5.3, at 4°C to remove detergent. The dialysate was again centrifuged at 10,000*g* at 4°C for 10 min after which the supernatant was made 60% saturated with ammonium sulfate and stirred for an hour at room temperature. The pellet from centrifugation of that mixture (10,000*g*; 4°C; 10 min)

was suspended in 600 ml of 0.1 *M* Tris–acetate buffer, pH 7.4, and stirred for one hour at room temperature with 30 g Sepharose 4B–concanavalin A (*12*). All arylsulfatase activity was bound to this glycoprotein affinity gel.

The Sepharose gel was isolated by filtration and washed copiously with 0.1 *M* Tris-acetate buffer, pH 7.4. The sulfhydrolases were then eluted with the same buffer containing 10% α-methylglucoside (Sigma Chemical Co.) and the elute concentrated to 60 ml against polyethylene glycol (Sigma) at 4°C. The enzyme solution was then dialyzed against 1 m*M* Tris–acetate, pH 7.1, at 4°C. The solution was adjusted to a pH of 6.0 with concentrated Tris–acetate and was applied to a column (2 × 12.5 cm) of Blue Sepharose CL-6B (Pharmacia Ltd.) equilibrated with Tris–acetate, 10 m*M*, pH 6.0 (*13*). Washing with the same buffer eluted the B enzyme; the A was isolated after washing with 10 m*M* Tris–acetate of pH 7.4.

Gel electrophoresis (*14*) showed that the A sulfatase migrated more slowly than did the B form (*7*) and that each was a single enzyme.

Mammalian arylsulfatases A and B were isolated from bovine liver obtained directly following slaughter. The procedures used for isolating and separating the two enzymes were those described above (see also (*12, 13*)). The sulfhydrolases so obtained were homogeneous on disc gel electrophoresis, the A form again migrating more slowly than the B to the negative pole. While the A form was highly reactive to *p*-nitrocatechol sulfate, and was inhibited by Cl^- but not by Ag^+, the B enzyme was comparatively unreactive to that substrate, was not inhibited by Cl^- and was by Ag^+. These characteristics define the commonly recognized differences between these two forms of the mammalian arylsulfatases (*4*).

RESULTS AND DISCUSSION

Table 1 contains the kinetic parameters for the reactions of a series of arylsulfate substrates with the arylsulfatase B from chicken liver. It is apparent from these

TABLE 1

KINETIC PARAMETERS FOR ARYLSULFATE SUBSTRATES: REACTION WITH ARYLSULFATASE B FROM CHICKEN LIVER IN 0.5 *M* AcO^-, pH 5.5, AT 25°C

Substrate	K_m(m*M*)[a]	V(10^7 *M* sec^{-1})[a]	V/K_m(10^4 sec^{-1})	k_0(10^4 sec^{-1})[b]
4-Nitrocatechol sulfate	2.9	12.5	4.3	3.72
4-Methylcatechol sulfate	143	1.0	0.07	—
4-Hydroxy-2-nitrophenyl sulfate	113	1.1	0.10	—
4-*tert.* Butylphenyl sulfate	50	2.5	0.05	—
4-Phenylphenyl sulfate	3.3	0.8	0.24	—
4-Fluorophenyl sulfate	2.7	0.1	0.04	—
3-Bromophenyl sulfate	0.3	0.2	0.67	—
4-Nitrophenyl sulfate	0.5	0.2	0.40	0.56

[a] Values determined from initial velocity experiments.

[b] Values determined from pseudo first-order kinetics.

results that there are no electronic substituent or bulk effects. In addition, hydrophobicity parameters do not indicate any simple substituent correlation with either of the Michaelis constants. The data also show that the direct determinations of the ratios V/K_m as the pseudo-first-order rate constant k_ψ for *p*-NCS and *p*-NPS agree well with the calculated values from the measurements of K_m and V. This correspondence indicates the absence of substrate or product inhibitions, as initial rates measured for the estimation of the Michaelis–Menten constants were made at substrate concentrations different from those used in measuring k_ψ ([*p*-NCS] = 5.6×10^{-5}–2.0×10^{-4} *M*; [*p*-NPS] = 6.3×10^{-5}–5.0×10^{-4} *M*). A further demonstration of the lack of correlation between the structure of substrates and their reactivities with the arylsulfatase was found with a series of substituted naphthyl sulfates, where values of k_ψ varied over a narrow range in a random manner (unpublished data).

Arylsulfatase A from chicken liver resembles the B enzyme in several ways, as can be concluded from the results collected in Tables 2 and 3. Again there are no obvious substituent effects in any of the kinetic parameters. The pH/rate profile for the A form is also similar to that for the B enzyme; the maxima are at pH 5.6 in typical bell-shaped curves.

However, there are several differences in reactivities between the two enzymes considered here. While the arylsulfatase A reacted with 1-naphthyl sulfate and did not hydrolyze the 2-naphthyl analog, the B form reacted with that latter sulfate but not the former. Additional differences were found in the effects of Ag^+ and Cl^- ions. Under identical conditions arylsulfatase A is almost completely inhibited by Ag^+ (3×10^{-4} *M*), while Cl^- has no significant effect on its activity; the arylsulfatase B enzyme is unaffected by Ag^+, but is by Cl^- (about 65% inactivation). This behavior parallels that of the analogous arylsulfatases from mammals (*2*).

It is apparent from Table 2 that the calculated ratios of V/K_m for several of the substrates examined (for example *p*-nitrocatechol and *p*-methylcatechol sulfates)

TABLE 2

KINETIC PARAMETERS FOR ARYLSULFATASE A FROM CHICKEN LIVER HYDROLYSIS OF ARYLSULFATE SUBSTRATES IN 0.5 *M* AcO^-, pH 5.5, AT 25°C

Substrate	K_m(m*M*)[a]	V(10^7 *M* sec^{-1})[a]	V/K_m(10^4 sec^{-1})	k_ψ(10^4 sec^{-1})[b]
4-Nitrocatechol sulfate	0.2	2.1	10.50	2.03
4-Methylcatechol sulfate	0.3	3.4	11.33	3.60
4-Hydroxy-2-nitrophenyl sulfate	2.4	2.4	1.00	0.76
4-*tert*. Butylphenyl sulfate	0.2	0.5	2.50	5.25
4-Fluorophenyl sulfate	0.3	0.4	1.33	2.26
3-Bromophenyl sulfate	0.1	0.5	5.00	1.18
3-Nitrophenyl sulfate	0.03	0.05	1.67	1.57
4-Nitrophenyl sulfate	0.6	0.2	0.33	0.50
4-Phenylphenyl sulfate	0.1	0.6	6.00	1.39

[a] Values determined from initial velocity experiments.
[b] Values determined from pseudo first-order kinetics.

TABLE 3

EFFECT OF THE VARIATION OF INITIAL *p*-NITROCATECHOL SULFATE CONCENTRATION ON THE PSEUDO FIRST-ORDER RATE CONSTANT (k_ψ) FOR ARYLSULFATASE A FROM CHICKEN LIVER IN 0.5 *M* AcO^-, pH 5.5, AT 25°C

[*p*-NCS](10^4 *M*)	k_ψ(10^4 sec^{-1})	k_{calc}(10^4 sec^{-1})[b]
1[a]	7.00	6.90
2	5.00	5.20
4	3.80	3.50
10	1.50	1.70
20	1.00	0.95

[a] After reaction was over, another *p*-NCS aliquot was added and for this reaction k_ψ was found to be 4.2×10^{-4} sec^{-1}.

[b] k_{calc} are obtained using Eq. [1] where $V/K_m = 10.3 \times 10^{-4}$ sec^{-1} and $K_I^{app} = 2.04 \times 10^{-4}$ *M*.

do not agree with the experimentally determined pseudo first-order rate constants (k_ψ) suggesting that substrates, products, or a combination of these may be inhibitory for the arylsulfatase A from chicken liver. It was with this enzyme that Fedecka-Bruner and Croisille (*7*) noted anomalous behavior while following the time course of the reaction with *p*-nitrocatechol sulfate, suggesting the possibility of analogy with the mammalian A enzyme (*2*). Thus it was of interest to examine the reaction of this arylsulfatase further.

In Table 3 are shown the results from experiments performed at varying initial *p*-nitrocatechol sulfate concentrations. It is seen that k_ψ decreases as the initial substrate concentration increases, possibly the result of either substrate or product inhibition. When aliquots of the catechol sulfate were added successively to completed hydrolyses of the preceding aliquots, data similar to those of Table 3 were accumulated. Thus there is apparent inhibition by product(s) without any evidence of substrate inhibition. Independent experiments showed that k_ψ varies in a linear fashion when the enzyme concentration was changed over a fourfold range. It is, then, reasonable to conclude that polymerization of the enzyme is not a factor in the data discussed here.

As there are two products from the reaction (*p*-nitrocatechol and SO_4^{2-}), only an apparent inhibition constant (K_I^{app}) can be calculated from the data of Table 3. This was done as described by Niemann and co-workers (*15, 16*). K_m and V were respectively determined as 1.94×10^{-4} *M* and 2×10^{-7} *M* sec^{-1}. From these values, K_I^{app} was calculated (*15, 16*) as 2.04×10^{-4} *M*. Experiments in which the initial substrate concentration was varied (2×10^{-3}–2×10^{-2} *M*) showed that V remained constant at 2×10^{-7} *M* sec^{-1} while K_I^{app} varied (8×10^{-4}–7×10^{-3} *M*) which indicates that the inhibition is of the competitive type.

Calculated first-order rate constants (k_{calc}) were obtained using the expression (*15, 16*)

$$k_{calc} = \frac{V/K_m}{1 + s_0/K_I^{app}}. \qquad [1]$$

where $V/K_m = 10.3 \times 10^{-4}$ sec^{-1}, $K_i^{app} = 2.04 \times 10^{-4}$ M, s_0 = the relevant substrate concentration. The values so obtained are shown in the last column of Table 3 and are in good agreement with the experimentally determined values of k_ψ. Such agreement must indicate that the phenomenon discussed here is one of product inhibition.

Individual inhibition constants were obtained from experiments in which concentrations of SO_4^{2-} and of *p*-nitrocatechol at zero time were varied. The data are presented in Table 4. From them individual inhibition constants were calculated: $K_i^{SO_4^{2-}} = 1.39 \times 10^{-3}$ M and $K_i^{p\text{-}NO_2\text{catechol}} = 2.18 \times 10^{-4}$ M.

The data discussed here clearly suggest that the anomalous behavior by the arylsulfatase A of chicken liver is completely attributable to product inhibition. The work described here was performed at 25°C and at substrate concentrations lower than those commonly used with the mammalian A enzyme. To facilitate comparison with that sulfatase the temperature and substrate concentration were adjusted to those latter conditions (40°C; [*p*-NCS] = 4×10^{-3} M). However, the results with the avian A enzyme, which were in nature similar to those described above, clearly showed that only product inhibition was again operating, and constants were estimated as before: $K_i^{SO_4^{2-}} = 3.6 \times 10^{-3}$ M; $K_i^{p\text{-}NO_2\text{catechol}} = 4.5 \times 10^{-4}$ M. Thus it may be concluded that the arylsulfhydrolase A from chicken liver does not show the kinetic anomalies found with the analogous enzyme from mammalian sources (see (*2*) and below) and attributed to substrate-induced enzyme inactivation (*9*). The avian enzyme does, however, resemble that from mammals in its inhibition by products and by Ag^+.

Figure 1 shows a typical progress curve for the reaction of *p*-nitrocatechol sulfate in the presence of arylsulfatase A from bovine liver, substrate concentration being 5 mM and a generally employed "static" assay system being used (*4*). It is apparent that this is not a conventional kinetic plot in that the rate of the reaction diminishes and, after a time, increases again, a type of result commonly reported for the behavior of arylsulfatase A of mammals (*4*). However, when the reaction was performed at 0.1 mM substrate concentration and the progress directly followed spectrophotometrically (Fig. 1b), normal first-order rate con-

TABLE 4

EFFECT OF VARIATION OF INITIAL SULFATE ION AND *p*-NITROCATECHOL CONCENTRATIONS ON THE RATE OF HYDROLYSIS OF *p*-NCS BY ARYLSULFATASE A FROM CHICKEN LIVER IN 0.5 M AcO^-, pH 5.5, AT 25°C[a]

$[SO_4^{2-}](10^4\ M)$	[*p*-Nitrocatechol]$(10^4\ M)$	$k_\psi(10^4$ sec$^{-1})$
2	2	4.85
12	2	3.74
22	2	2.16
2	5	3.0
2	10	1.8

[a] [*p*-NCS] and [enzyme] were kept constant.

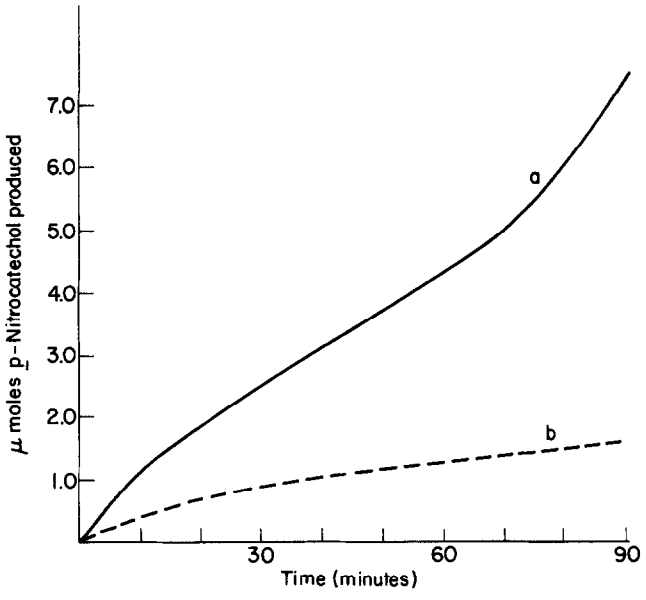

FIG. 1. Production of *p*-nitrocatechol versus time for the hydrolysis of *p*-nitrocatechol sulfate in 0.5 *M* acetate, pH 5.5, with bovine liver arylsulfatase A. The full line (a) was obtained when [substrate] = 5 m*M*; the broken line (b) when [substrate] = 0.1 m*M*.

stants were obtained, with linearity in the semilogarithmic plot over two or three half-lives. These results show that the anomalies with the nitrocatechol sulfate are dependent on the concentration of the substrate—when the latter is not saturating the enzyme, typical pseudo first-order kinetics may be obtained. With the procedures employed in the work reported here (*8*) at the substrate concentrations used (1×10^{-4}–1×10^{-5} *M*), none of the variety of the sulfates examined (Table 5) showed anomalies comparable with those of Fig. 1a; conventional kinetic results were obtained throughout.

Michaelis–Menten parameters K_m and V were estimated from double-reciprocal plots, and the pseudo-first-order constant k_ψ was independently measured. The results are collected in Table 5. When equivalence between the ratio V/K_m and k_ψ was not found, with for example *p*-phenyl and *p*-bromophenyl sulfates, the simplest explanation is of the occurrence of product inhibition, which has been previously described as occurring with mammalian arylsulfatase A ((*4*); compare above). The results of Table 5 show also that the product inhibition discussed is less important with *p*-methylcatechol, 1-bromo-2-naphthyl, and 4-hydroxy-2-naphthyl sulfates than elsewhere among the data presented here, since the agreements between k_ψ and V/K_m are within an acceptable range. No clear correlation could be found for K_m, V, of k_ψ with electronic (Hammett σ), hydrophobic (*17*), or steric characteristics of the variety of substrates examined.

Table 6 contains the results for hydrolysis, in the presence of arylsulfatase B from bovine liver, of members of the series of substrates employed with the A enzyme. Some differences between the mammalian A and B enzymes are apparent; for example *p*-fluorophenyl sulfate reacts readily with the B, but barely

TABLE 5

Michaelis Parameters and Pseudo-First-Order Rate Constants (k_ψ) for Hydrolyses of Arylsulfates in the Presence of Bovine Liver Arylsulfatase A

Substrate	K_m(mM)	V(10^4 M sec^{-1})	V/K_m(10^4 sec^{-1})	k_ψ(10^4 sec^{-1})
p-Nitrocatechol sulfate	2.68	4.91	1.83	1.94
p-Methylcatechol sulfate	33.3	16.7	0.5	4.14
p-Nitrophenyl sulfate	0.78	0.34	0.78	4.5
m-Nitrophenyl sulfate	0.13	0.18	1.20	0.79
m-Bromophenyl sulfate	2.13	0.73	0.34	1.04
p-Fluorophenyl sulfate	Very slow reaction			
p-tert. Butylphenyl sulfate	1.30	0.36	0.28	Very small
p-Phenylphenyl sulfate	6.39	3.67	0.56	0.21
1-Bromo-2-naphthyl sulfate	2.70	2.64	0.98	0.79
4-Hydroxy-2-naphthyl sulfate	6.67	2.25	0.34	0.43
6-Bromo-2-naphthyl sulfate	1.31	8.72	6.68	2.77
2-Naphthyl sulfate	0.37	0.25	0.70	0.30

measurably with the A arylsulfatase (Table 5). Again it was found that the data from the work with the B enzyme showed no simple relationship of kinetic parameters with electronic, hydrophobic, or steric factors for the substrates used.

A comparison of k_ψ with V/K_m in Table 6 shows that, as for the arylsulfatase A examined above, equivalence is not commonly found. It is probable that product inhibition is again occurring, as discussed above.

In summary, this study of four type II arylsulfhydrolases from avian and mammalian sources shows that the mammalian A enzyme is unique in displaying substrate inhibition in the presence of saturating concentrations of *p*-nitrocatechol sulfate (*4, 9*). Product inhibition was evident with both A enzymes examined here, and with the mammalian B arylsulfatase. With none of the arylsulfhydrolases studied in this work using a range of arylsulfate substrates was there evidence of correlation between Michaelis–Menten parameters and electronic, hydrophobicity, or steric factors. In this respect the type II and type I arylsulfatases examined here and elsewhere are similar (*6, 18*).

TABLE 6

KINETIC CONSTANTS FOR HYDROLYSIS OF VARIOUS ARYLSULFATES IN THE PRESENCE OF BOVINE ARYLSULFATASE B

Substrate	k_m(mM)	V(10^4 M sec^{-1})	V/K_m(10^4 sec^{-1})	k_ψ(10^4 sec^{-1})
p-Nitrocatechol sulfate	0.10	0.21	2.23	0.25
p-Methylcatechol sulfate	0.33	9.04	26.95	2.95
p-Fluorophenyl sulfate	0.02	0.57	24.25	4.49
p-Nitrophenyl sulfate	0.93	0.20	0.21	0.41
m-Bromophenyl sulfate	2.95	13.10	4.44	4.07
p-t-Butylphenyl sulfate	0.61	5.18	8.50	0.61

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