The Biochemistry of Aromatic Amines

THE RESISTANCE OF SOME *o*-AMINOARYL SULPHATES TO HYDROLYSIS BY ARYL SULPHATASES

By E. BOYLAND, D. MANSON, P. SIMS AND D. C. WILLIAMS

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, S.W. 3

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Aromatic amines are oxidized in animal tissues to *o*- and *p*-aminophenols, which are rapidly conjugated to give either sulphates or glucosiduronates. Some of the carcinogenic aromatic amines appear to produce their biological effects after conversion into *o*-aminophenols. 2-Naphthylamine, for example, which produces bladder cancer, is oxidized to 2-amino-6-naphthol and 2-amino-1-naphthol by liver slices, but these products are rapidly conjugated to yield sulphates and glucosiduronates in liver tissue (Booth, Boyland & Manson, 1955). Whereas 2-amino-6-naphthyl sulphate was hydrolysed by rat kidney, 2-amino-1-naphthyl sulphate was not hydrolysed by any of the tissues which were examined. One would expect that the conjugated forms of aminophenols excreted in urine would be hydrolysed by the glucuronidase and sulphatase which are present in almost all urines (Boyland, Wallace & Williams, 1955). The glucosiduronates of both 2-amino-6-naphthol and 2-amino-1-naphthol, and 2-amino-6-naphthyl sulphate are hydrolysed enzymically, but 2-amino-1-naphthyl sulphate is hydrolysed neither by urinary nor by any other sulphatases. Some other *o*-aminoaryl sulphates are resistant to hydrolysis by sulphatase, but all other aryl sulphates which have been examined are hydrolysed by aryl sulphatases. The aminophenyl sulphates derived from carcinogenic aromatic amines are generally resistant to the hydrolytic action of sulphatases.

Although 2-naphthylamine is carcinogenic when given to dogs, it is not active when implanted in the mouse bladder under conditions in which 2-amino-1-naphthol induces bladder cancer (Bonser, Clayson, Jull & Pyrah, 1952). When tested under the same conditions 2-amino-1-naphthyl sulphate did not induce bladder cancer (Bonser, Clayson & Jull, 1954).

METHODS

Materials. *o*-Aminophenyl sulphates were prepared by persulphate oxidation of the corresponding aromatic amines (cf. Boyland, Manson & Sims, 1953; Boyland & Sims, 1954). Potassium indoxyl sulphate was prepared by the action of potassium persulphate on indole (Boyland, Sims & Williams, unpublished work). Other aminophenyl sulphates and the nitrophenyl sulphates were prepared from the correspond-

ing nitrophenols by the method of Burkhardt & Wood (1929), and the dipotassium salt of 2-hydroxy-5-nitrophenyl sulphate was prepared by the action of persulphate on *p*-nitrophenol (Roy, 1953). Potassium 1-acetamido-2-naphthyl sulphate, prepared by the action of acetic anhydride on potassium 1-amino-2-naphthyl sulphate, separated from 90% aqueous ethanol-ether in clusters of leaflets. (Found: C, 45.2; H, 3.3; N, 4.45. $C_{12}H_{10}O_5NSK$ requires C, 45.1; H, 3.2; N, 4.4%) 2-Amino-1-naphthyl phosphate was prepared by oxidation of 2-naphthylamine with perphosphoric acid in the presence of acetone (Boyland & Manson, unpublished work). 2-Amino-1-naphthyl glucosiduronate was prepared by the acid hydrolysis of 2-acetamido-1-naphthyl glucosiduronate, which was isolated from the urine of rabbits dosed with 2-acetamido-1-naphthol. Taka diastase was obtained from Parke Davis Ltd. Acid phosphatase, alkaline phosphatase and bacterial β -glucuronidase were obtained from Sigma Chemical Co., Missouri, U.S.A. Spleen β -glucuronidase was supplied by Viobin Laboratories, Stamford, Conn., U.S.A.

Paper chromatography. The descending technique (Consden, Gordon & Martin, 1944) was used on Whatman no. 1 chromatography paper with the organic phase from a freshly prepared mixture of butanol, acetic acid and water (4:1:5) (Partridge, 1946). The chromatograms were run for 18 hr. at room temperature and then examined either under a Hanovia 'Chromatolite' ultraviolet lamp directly or in daylight after spraying with a solution of *p*-dimethylaminobenzaldehyde (0.5% in 10% aqueous ethanol containing 2.5% HCl).

Hydrolysis by sulphatase. (a) *Taka diastase.* Solutions of the substrates (2 ml., 0.01 M), aqueous Taka diastase (1 ml., 0.5%) and acetate buffer (2 ml., 0.2 M, pH 6.0) were mixed in stoppered tubes and incubated for 18 hr. at 37°. The hydrolysis of each substrate was carried out in duplicate and a blank, without Taka diastase, was also incubated. Taka diastase solution (1 ml.) was added to the blank, and the phenol liberated in any of the three solutions was estimated in one of two ways.

Method 1. Samples (1 ml.) of each of the three solutions were added to tubes each containing aqueous Na_2CO_3 (3 ml., 20%, w/v), and Folin and Ciocalteu's reagent (1 ml.) was added to each tube. The tubes were centrifuged for 15 min. at 600 g and the absorption of the solutions at 700 m μ . was measured with a Unicam SP. 500 spectrophotometer. A standard was also set up by treating an aqueous solution of the corresponding phenol (0.01 M) in the same way. The results were expressed as $\mu g.$ of the sulphate hydrolysed/unit of sulphatase activity/hr. The unit of sulphatase is that quantity which, when present in 1 ml. of water and incubated at 37° with 4-nitrocatechol sulphate (1 ml., 0.05 M)

and acetate buffer (1 ml., 0.2M, pH 6.0), releases 1 μ g. of nitrocatechol/hr.

Method 2. (a) Samples (0.3 ml.) of each of the three solutions were added to aqueous Na_2CO_3 (2 ml., 20%, w/v) and diazotized sulphanilic acid (2 ml., 9 parts of 0.5% aqueous sulphanilic acid and 1 part of 1% NaNO_2 solution kept at 0°) was added. The solutions were kept for 15 min. at room temperature and the colour was estimated on a Unicam SP. 500 spectrophotometer by comparison with the colour produced by the phenol. In cases where both methods were used, good agreement was obtained.

(b) *Urine*. The procedure was the same except that urine of known high sulphatase activity was used in place of the Taka diastase solution.

Hydrolysis of 2-amino-1-naphthyl glucosiduronate by β -glucuronidase. Aqueous 2-amino-1-naphthyl glucosiduronate (2 ml., 0.01M) was incubated at 37° in acetate buffer (2 ml., pH 6.0) with bacterial β -glucuronidase and in acetate buffer (2 ml., pH 4.5) with calf-spleen β -glucuronidase overnight. Free 2-amino-1-naphthol was detected as before.

RESULTS

*Aryl sulphates without *o*-amino groups*. The hydrolysis rates and R_F values of the aryl sulphuric esters, without amino groups *ortho* to the ester groups, are shown in Table 1. The four nitrophenol derivatives are hydrolysed readily, both by the

Table 1. *Properties of aryl sulphates*

Sulphate of	R_F value		Hydrolysis			Apparent pK_a
	Phenol	Ester	Taka diastase (μ g./unit/hr.)	Urine sulphatase (μ g./unit/hr.)	5N-HCl at 37° (1/time for half hydrolysis)	
<i>o</i> -Nitrophenol	—	—	215	129	0.125	—
<i>m</i> -Nitrophenol	0.91	0.32	294	217	0.092	—
<i>p</i> -Nitrophenol	0.88	0.35	480	113	0.333	—
2-Hydroxy-5-nitrophenol	—	—	270	248	0.025	6.8
<i>m</i> -Aminophenol	—	—	32	23	0.016	4.1
<i>p</i> -Aminophenol	—	—	62	81	0.192	4.4
1-Naphthol	0.92	0.46	290	310	0.410	—
2-Naphthol	—	—	117	92	—	—
2-Amino-6-naphthol	0.79	0.28	138	178	0.001	—
2-Acetamido-6-naphthol	—	—	46	48	0.014	—
Indoxyl	—	0.25	11	9.4	—	—

Hydrolysis by acid. All compounds tested were easily hydrolysed in N-HCl at 100°. Solutions of the substrates (5 ml., 0.01M) and 10N-HCl (5 ml.) were heated to 37° separately and then mixed, thoroughly shaken and incubated at 37°. Samples (0.3 ml.) were removed at intervals of 2, 5, 10, 15, 20, 30, 40, 50 and 60 min. and immediately added to aqueous Na_2CO_3 (2 ml., 20%, w/v). These solutions were treated with diazotized sulphanilic acid and the liberated phenols were estimated as before. The times for half hydrolysis were calculated from the hydrolysis curves and the results are expressed as reciprocals (i.e. the rates of hydrolysis) of these figures.

Determination of pK_a values. These were determined by potentiometric titrations of the sulphates at 25°. In most cases the sodium or potassium salt (100–200 mg.) in water (10–25 ml.) was titrated with 0.1N-HCl and the pH of the solution was measured at 0.25 ml. intervals with a Cambridge pH meter, using a glass electrode, pK_a being taken as the pH at which half the acid was neutralized. In a few cases, where the salt was not available, the acid was titrated against 0.1N-NaOH.

Hydrolysis of 2-amino-1-naphthyl phosphate. Aqueous 2-amino-1-naphthyl phosphate (2 ml., 0.01M) was incubated with acid phosphatase at 37° in acetate buffer (2 ml., pH 5.0) and with alkaline phosphatase in bicarbonate buffer (2 ml., pH 10.0) for 1 hr. Free 2-amino-1-naphthol was detected by the formation of a dye when the incubated solutions were shaken with aqueous 2N- NH_3 . The dye was extracted by benzene, forming a characteristic purple solution.

sulphatases of urine and of Taka diastase. All three isomeric nitrophenyl sulphates are hydrolysed more rapidly by Taka diastase preparation than by the enzyme from urine; the hydrolysis with Taka diastase proceeds most rapidly with the *p*-isomer and least rapidly with the *o*-compound. The *m*- and *p*-aminophenyl sulphates are hydrolysed much more slowly than the corresponding nitrophenyl sulphates. The enzymic hydrolysis of 1-naphthyl sulphate proceeds at more than twice the rate of that of 2-naphthyl sulphate.

*Aryl sulphates with *o*-amino groups*. Table 2 shows that eight of the aryl sulphates with *o*-amino groups are not hydrolysed by sulphatase. The sulphates which are resistant to hydrolysis all have apparent pK_a values of 4.3 or higher. The hydrolysis rate of the other sulphates increases as the pK_a value decreases so that the least basic of the esters, that of 2-amino-4-chlorophenol (pK_a 2.2) is hydrolysed almost as rapidly as esters which have no basic groups. The substitution of a dimethyl-amino group in place of an amino group raises the pK_a by approximately 2 units. In the cases of the sulphates of 2-aminophenol and 2-dimethylaminophenol, and of 1-amino-2-naphthol and 1-dimethyl-amino-2-naphthol, the limiting pK_a value of 4.3 lies between the pK_a values of the methylated and

Table 2. *Properties of o-aminoaryl sulphates*

Sulphate of	R_F value		Hydrolysis			Apparent pK_a
	Phenol	Ester	Taka diastase ($\mu\text{g./unit/hr.}$)	Urine sulphatase ($\mu\text{g./unit/hr.}$)	5N-HCl at 37° (1/time for half hydrolysis)	
2'-Chloro-4-dimethylamino-3-hydroxystilbene	0.72	0.41	0	0	0.68	—
4-Dimethylamino-3-hydroxy-2'-methylstilbene	0.65	0.32	0	0	0.333	7.3
2-Dimethylamino-1-naphthol	—	0.65	0	0	0.112	6.9
4-Dimethylamino-3-hydroxydiphenyl	—	—	0	0	0.046	6.6
2-Dimethylaminophenol	0.81	0.53	0	0	0.059	5.7
1-Dimethylamino-2-naphthol	—	0.64	0	0	0.153	4.8
4-Amino-3-hydroxydiphenyl	0.84	0.61	0	0	0.078	4.5
2-Amino-1-naphthol	—	0.52	0	0	0.083	4.3
2-Amino-4:5-dimethylphenol	0.68	0.53	22	23	0.087	4.2
2-Amino-5-methylphenol	0.87	0.60	41	43	0.100	3.9
2-Aminophenol	0.69	0.39	36	40	0.385	3.7
2-Amino-4-methylphenol	0.86	0.59	8	7	0.400	3.7
2-Amino-3:6-dimethylphenol	—	—	45	45	0.062	3.6
2-Methylaminophenol	0.75	0.51	16	11	0.264	3.6
2-Amino-3-methylphenol	0.83	0.51	32	44	0.125	3.3
1-Amino-2-naphthol	0.75	0.59	22	29	0.125	2.9
2-Amino-3-carboxyphenol	0.89	0.30	33	31	0.166	—
2-Amino-5-sulphophenol	0.11	0.30	33	25	0.222	—
3-Hydroxykynurenine	0.20	0.04	50	41	0.252	—
2-Amino-5-carboxyphenol	0.67	0.16	56	68	0.154	—
2-Amino-5-bromophenol	0.74	0.62	66	56	0.500	2.7
1-Acetamido-2-naphthol	—	—	8	71	0.133	—
2-Acetamido-1-naphthol	—	—	98	138	1.08	—
2-Acetamidophenol	—	—	131	167	0.210	—
2-Amino-4-chlorophenol	0.82	0.37	154	170	0.006	2.2

unmethylated compounds: the unmethylated but not the methylated compounds were hydrolysed by sulphatase. While there is reasonable correlation between the hydrolysis by sulphatase and the pK_a of the amino groups, there is no correlation between the rates of enzymic hydrolysis and of acid hydrolysis.

Aliphatic sulphates. Neither of the sulphatase preparations hydrolysed ethyl sulphate or 2-aminoethyl sulphate, in agreement with the findings of Neuberg & Wagner (1925).

Effect of pH and substrate concentration. When 2-amino-1-naphthyl sulphate was incubated with the sulphatases of urine and of Taka diastase at pH 3.0, 4.5, 5.8, 7.0, 8.0, 9.1 and 10.5 for 48 hr. at 37° no detectable hydrolysis occurred. Urinary sulphatase was incubated with 0.001, 0.01 and 0.1M 2-amino-1-naphthyl sulphate at pH 6.0. No hydrolytic action was detected.

Inhibition experiments. Urinary sulphatase activity on 2-hydroxy-5-nitrophenyl sulphate, as described by Roy (1953), was measured alone and in the presence of 0.1, 0.01 and 0.001M 2-amino-1-naphthyl sulphate, and in another experiment in the presence of 0.01M 2-amino-1-naphthol. These substances did not reduce the rate of hydrolysis of the nitrophenyl sulphate.

Digestive juice of snail. The digestive juice of *Helix pomatia*, which contained 4600 units of aryl sulphatase/ml., did not hydrolyse 2-amino-1-naphthyl sulphate.

DISCUSSION

The complete resistance of some *o*-aminophenyl sulphates to hydrolysis by sulphatase is surprising. The more basic esters, which are not hydrolysed, will exist to a greater extent in the zwitterion form at pH 5.8, the optimum pH for the enzyme. In the case of 2-dimethylaminophenyl sulphate, which has a pK_a of 5.7, almost equal amounts of the compound will be in the form of the acid ion (I) and the zwitterion (II).

The *o*-aminophenyl sulphates which are hydrolysed by sulphatase have weak basic groups, which will be largely ionized at the pH at which the enzyme is active.

Of the eight *o*-aminoaryl sulphuric esters which are resistant to enzymic hydrolysis, five are derivatives of carcinogenic aromatic amines. The carcinogenic amines are 2'-chloro-4-dimethylaminostilbene and 4-dimethylamino-2'-methylstilbene (Haddow, Harris, Kon & Roe, 1948), 4-dimethylaminodiphenyl (Miller, Miller, Sandin & Brown, 1949), 4-aminodiphenyl (Walpole, Williams & Roberts, 1952) and 2-naphthylamine (Hueper & Wolfe, 1937). The bases from which the other resistant esters are derived, i.e. 2-dimethylnaphthylamine, 1-dimethylnaphthylamine and dimethylaniline, do not appear to have been tested for carcinogenic activity. Of the bases giving *o*-aminoaryl sulphuric esters, which have low pK_a values and are hydrolysed by sulphatases, none are known to be carcinogenic.

That part of the dose of any carcinogenic aromatic amine which is oxidized to an *o*-aminophenol and then converted into an aryl sulphuric ester will presumably be inactivated, but the portion of the aminophenol which is conjugated with glucosiduronic acid could yield free carcinogenic *o*-aminophenol in any tissue containing glucuronidase. These observations are of interest in relation to the experiments of Elson, Goulden & Warren (unpublished work), which showed that carcinogenic aromatic amines caused increase in glucosiduronic acid excretion, while the two non-carcinogenic amines examined tended to increase the level of sulphuric ester excretion.

The carcinogenic action of the aromatic amines might therefore be reduced by inhibition of (1) the oxidation to carcinogenic *o*-aminophenols, (2) the conjugation of the carcinogenic *o*-aminophenols with glucosiduronic acid (assuming that this inhibition would be associated with increased formation of the inert sulphuric ester), or (3) glucuronidase of the tissues and body fluids.

SUMMARY

1. Sulphatase preparations from human urine or Taka diastase hydrolyse all of a number of aryl sulphates except those with a basic amino group in the *ortho* position to the ester group.

2. No *o*-aminoaryl sulphates with pK_a values of 4.3 or higher are hydrolysed, and the rates of

hydrolysis of a series of less basic aminophenyl sulphates increase as the basicity of the amino groups decreases.

3. The *o*-aminophenyl sulphates derived from carcinogenic aromatic amines have relatively strong basic groups and are not hydrolysed by sulphates.

4. 2-Amino-1-naphthyl sulphate, which is not hydrolysed by sulphatase, does not inhibit the hydrolysis of 2-hydroxy-4-nitrophenyl sulphate.

5. 2-Amino-1-naphthyl phosphate is hydrolysed by acid and alkaline phosphatase and 2-amino-1-naphthyl glucosiduronate is hydrolysed by β -glucuronidase.

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