Studies in Detoxication

43. A STUDY OF THE ARYLSULPHATASE ACTIVITY OF TAKADIASTASE TOWARDS SOME PHENOLIC ETHEREAL SULPHATES

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Several types of sulphatases occur in nature. These are distinguished by the nature of the ester group found in the ethereal sulphates which they hydrolyse (Fromageot, 1950). Takadiastase, a commercial preparation of *Aspergillus oryzae*, possesses arylsulphatase (phenolsulphatase) activity (Neuberg & Kurono, 1923), for it hydrolyses phenolic monoesters of sulphuric acid but has no action on aliphatic sulphuric esters. Others preparations of *A. oryzae* such as 'Mylase P' (Abbott, 1947) and 'Clarase' (Dzialoszynski, 1947) are also active. Arylsulphatase also occurs in animal tissues, especially liver, and according to Huggins & Smith (1947) rat neoplasms contain significantly higher amounts of the enzyme than normal tissues.

Mylase P has been shown to release phenolic oestrogens from their conjugates in pregnant mares' urine in yields 20% higher than those obtained by acid hydrolysis (Cohen & Bates, 1949). The use of sulphatase thus provides a gentle method of hydrolysis for ethereal sulphates where acid hydrolysis might prove destructive. We required sources of arylsulphatase for this purpose, and the present work was undertaken to provide possible methods of assay of arylsulphatase and to obtain information about its activity towards different phenolic sulphuric esters.

Recent methods of arylsulphatase assay are those of Abbott (1947) using potassium phenylsulphate with the Folin-Ciocalteu reagent, and of Huggins & Smith (1947) using potassium *p*-nitrophenylsulphate as substrate (cf. Abbott & East, 1949). In the present work we have compared the action of takadiastase on the alkali salts of *o*-, *m*- and *p*-nitro-, *p*-chloro-, *p*-aldehydo-, 4-hydroxy-2-nitro-, 4-hydroxy-3-nitro- and 2-hydroxy-5-nitro-phenylsulphuric acids. In each case the liberated phenols were estimated in alkaline solution as their anions whose spectra were different from the corresponding undissociated phenols and ethereal sulphates. By this method each phenol could be estimated spectrophotometrically or colorimetrically in the presence of its ethereal sulphate, using a relatively short incubation time in the enzyme experiments.

EXPERIMENTAL

Spectroscopic measurements were made with a Unicam quartz spectrophotometer, Model SP500, colorimetric measurements with a Hilger Spekker absorptiometer and pH measurements with a Cambridge pH-meter.

Preparation of substrates. The potassium salts of *o*- and *p*-nitro-, *p*-chloro- and *p*-aldehydo-phenylsulphuric acids and the sodium salt of *m*-nitrophenylsulphuric acid were prepared according to Burkhardt & Lapworth (1926) and Burkhardt & Wood (1929). The potassium salts of 4-hydroxy-2-nitro-, 4-hydroxy-3-nitro- and 2-hydroxy-5-nitro-phenylsulphuric acids were prepared for the first time by one of us (Smith, 1951). These salts were purified by recrystallization from water or ethanol-water mixtures until free from inorganic sulphate, chloride and free phenols. Their purity was checked by analysis for Na or K gravimetrically, or for phenols spectroscopically.

Absorption spectra of the substrates and phenolic anions. These were determined in 0.1N-NaOH and are recorded in Table 1. Fig. 1 shows the spectra of 4-nitrocatechol and its monosulphate and illustrates the difference between the spectrum of a phenolic anion and that of its sulphate.

Preparation of the enzyme solution. Takadiastase (Parke, Davis and Co. Ltd.) was either dissolved in water, or in 0.5M-acetate buffer as required. These solutions were kept at 0°, but they deteriorated in about a week. The takadiastase used contained added lactose (reducing sugar 96%, calc. as lactose) and 0.4% N (Kjeldahl). This commercial preparation was thus largely lactose. It has been reported that phosphates inhibit sulphatase and therefore phosphate buffers were avoided. At the optimum pH of sulphatase (about pH 6), acetate buffers are not highly efficient, so that they were used at 0.5M strength to ensure their effectiveness.

The action of takadiastase on p-aldehydo- and p-chloro-phenylsulphates

Methods

(a) *Using potassium p-aldehydophenylsulphate.* To each of four tubes there were added 0.4 ml. of 0.5M-acetate buffer and 0.4 ml. enzyme solution in 0.5M-acetate buffer. To two of these tubes ('test') there was added 0.4 ml. of a solution of potassium *p*-aldehydophenylsulphate in 0.5M-acetate buffer of the same pH (all solutions having been previously warmed to 37°). The other two tubes were the controls. All tubes were stoppered and incubated for 1 hr. at 37°. After incubation, 0.4 ml. of substrate solution was added to the

Table 1. Absorption maxima of certain phenols and their ethereal sulphates in 0.1 N-NaOH

	$\lambda_{\max.}$	$\epsilon_{\max.}$	$\lambda_{\max.}$	$\epsilon_{\max.}$
<i>o</i> -Nitrophenol	233	3850	412-415	4500
Potassium <i>o</i> -nitrophenylsulphate	260	4400	—	—
<i>m</i> -Nitrophenol	290	4050	395	1450
Sodium <i>m</i> -nitrophenylsulphate	265	6500	—	—
<i>p</i> -Nitrophenol	230	4550	402	17 000
Potassium <i>p</i> -nitrophenylsulphate	280	9000	—	—
<i>p</i> -Chlorophenol	222	8900	245	11 650
			298	2600
Potassium <i>p</i> -chlorophenylsulphate	215	10 500	267.5	350
			275	300
<i>p</i> -Hydroxybenzaldehyde	237	7680	330	27 500
Potassium <i>p</i> -aldehydophenylsulphate	258	16 300	—	—
Nitroquinol	237	13 100	540	3850
Potassium 4-hydroxy-2-nitrophenylsulphate	235	17 055	380	1390
Potassium 4-hydroxy-3-nitrophenylsulphate	225	16 800	423	4750
	279	3900	—	—
4-Nitrocatechol	390-395	6300	510	11 300
Potassium 2-hydroxy-5-nitrophenylsulphate	405-410	18 150	—	—

two control tubes and 3.8 ml. of 0.15 N-NaOH to all four tubes. The extinctions of the tubes were then read at 330 $m\mu$. in the spectrophotometer, using 1 cm. cells, against a blank of 0.8 ml. buffer, 0.4 ml. water and 3.8 ml. 0.15 N-NaOH. The amount of *p*-hydroxybenzaldehyde liberated was then calculated from the relation:

$$p\text{-hydroxybenzaldehyde } (\mu\text{g.}) = \frac{(E_t - E_c) \times 122.1 \times 10^6 \times 5}{27\,000 \times 100} \\ = 22.2 (E_t - E_c),$$

where E_t and E_c are the observed spectrophotometer readings of the 'test' and 'control' tubes respectively at 330 $m\mu$; 122.1 is the molecular weight and 27 000 the $\epsilon_{\max.}$

Fig. 1. The ultraviolet absorption spectra of 4-nitrocatechol (full line) and potassium 2-hydroxy-5-nitrophenylsulphate (broken line) in 0.1 N-NaOH.

in alkali of *p*-hydroxybenzaldehyde. Control tubes of substrate and buffer were omitted because a separate experiment showed that no *p*-hydroxybenzaldehyde was liberated on incubating potassium *p*-aldehydophenylsulphate with acetate buffers from pH 4.1 to 6.7. The control tubes con-

taining enzyme and buffer, however, were included because the light absorption of the enzyme extracts may vary slightly on incubation with acetate buffer. At 330 $m\mu$. proteins absorb slightly, but in view of the very low protein content of takadiastase, the removal of protein was unnecessary. However, with preparations of arylsulphatase of high protein content, it may be necessary to remove protein as described in the next section.

(b) *Using potassium p-chlorophenylsulphate.* The procedure followed was similar to that described by Spencer & Williams (1951) for the assay of β -glucuronidase with *p*-chlorophenyl-glucuronide. In preliminary experiments it was found that potassium *p*-chlorophenylsulphate on incubation for 3 hr. with 0.5 M-acetate buffers, pH 4.1-6.7, yielded no spectrophotometrically detectable amount of *p*-chlorophenol. The procedure for the assay of the arylsulphatase activity of takadiastase was the following.

The reagents and procedure up to the incubation were as in section (a) above except that centrifuge tubes were used. After incubation for 2 or 3 hr., 0.4 ml. of substrate solution was added to the control tubes, and then 4.8 ml. of absolute ethanol to all four tubes. The tubes were centrifuged for 3 min. to remove any precipitated protein and 5 ml. of the clear supernatants transferred to tubes containing 5 ml. 0.2 N-NaOH. The extinctions of these solutions were then read at 245 $m\mu$. in the spectrophotometer against blanks made up of 0.4 ml. acetate buffer and 0.8 ml. water diluted with ethanol and 0.2 N-NaOH as for the test solutions. The amount of *p*-chlorophenol liberated is

$$\frac{(E_t - E_c) \times 128.5 \times 6 \times 10\,000}{11\,650 \times 5} = 132(E_t - E_c) \mu\text{g.}$$

A study of the recovery of *p*-chlorophenol measured at 245 $m\mu$. and of *p*-hydroxybenzaldehyde at 330 $m\mu$., showed that the maxima appeared to obey the Lambert-Beer Law, and that the phenols could be recovered over a wide range of concentrations to the extent of $100 \pm 3\%$.

Effect of pH and substrate concentration on the hydrolysis of potassium p-aldehydo- and p-chloro-phenylsulphate by takadiastase is shown in Table 2.

In optimum conditions with these two substrates, extent of hydrolysis was proportional to time up to 4 hr. and to the quantity of takadiastase present up to 0.5 %.

Table 2. *Investigation of effects of pH and substrate concentration on hydrolysis of substituted phenylsulphates by takadiastase*

(Temperature 37° throughout; other conditions noted in parentheses; taka = takadiastase.)

Substrate (all K salts unless otherwise stated)	Optimum pH	Optimum substrate concn. and K_m
<i>p</i> -Aldehydophenylsulphate	5.8 (little variation 5.5-6.1) (1% taka; 0.003M-substrate; 1 hr.)	$2.7 \times 10^{-3}M$ No inhibition up to $3 \times 10^{-2}M$ K_m , $2.1 \times 10^{-4}M$ (pH 5.8; 1% taka; 1 hr.)
<i>p</i> -Chlorophenylsulphate	6.15 (sharp maximum) (3% taka; 0.0125M-substrate; 3 hr.)	$1 \times 10^{-2}M$ No inhibition up to $3 \times 10^{-2}M$ K_m , $9.7 \times 10^{-4}M$ (pH 6.15; 3% taka; 3 hr.)
<i>o</i> -Nitrophenylsulphate	6.3 (little variation 6.0-6.5) (4% taka; 0.005M-substrate; 1 hr.)	$6 \times 10^{-3}M$ No inhibition up to $2 \times 10^{-2}M$ K_m , $3.4 \times 10^{-4}M$ (pH 6; 4% taka; 1 hr.)
<i>m</i> -Nitrophenylsulphate (Na salt)	6.2-6.4 (little variation 5.9-6.5) (4% taka; 0.005M-substrate; 2 hr.)	$6 \times 10^{-3}M$ No inhibition up to $2 \times 10^{-2}M$ K_m , $8.35 \times 10^{-4}M$ (pH 6; 4% taka; 2 hr.)
<i>p</i> -Nitrophenylsulphate	6.2 (little variation 6.0-6.4) (1% taka; 0.005M-substrate; 1 hr.)	$1.5 \times 10^{-2}M$ No inhibition up to $2 \times 10^{-2}M$ K_m , $1.7 \times 10^{-4}M$ (pH 6.1; 1% taka; 1 hr.)
4-Hydroxy-2-nitrophenylsulphate	6.0 (little variation 5.9-6.1) (2% taka; 0.002M-substrate; 2 hr.)	$2 \times 10^{-3}M$ No inhibition up to $5 \times 10^{-3}M$ K_m , $1.25 \times 10^{-4}M$ (pH 5.9; 2% taka; 2 hr.)
2-Hydroxy-5-nitrophenylsulphate	5.8-5.9 (2% taka; 0.0025M-substrate; 2 hr.)	$2.5 \times 10^{-3}M$ Inhibition beyond $2.5 \times 10^{-3}M$ K_m , $3.5 \times 10^{-4}M$ (pH 5.9; 2% taka; 2 hr.)

*The action of takadiastase on the o-, m- and p-nitrophenylsulphates**Method*

A solution (1 ml.) of the substrate dissolved in M-acetate buffer and the aqueous enzyme solution (1 ml.) were added to two tubes. These tubes, together with a control tube containing the substrate-buffer solution only, were incubated at 37° for the required period. Then 1 ml. of 0.33N-NaOH was added to all three tubes and 1 ml. of enzyme solution to the control tube. The extinctions of the contents of the first two tubes were then read in the spectrophotometer, with the contents of the control tube in the blank cell of the instrument. Readings were made at 412 m μ . for *o*-, at 395 m μ . for *m*- and at 402 m μ . for *p*-nitrophenol. The amount of nitrophenol in 3 ml. of test solution was then calculated from the relation, $\mu g.$ nitrophenol = Ex , where x is 92.66 for *o*-, 287.6 for *m*- and 24.5 for *p*-nitrophenol and E is the spectrophotometer reading at the appropriate wavelength, x being calculated as before from known ϵ_{max} and molecular weights of the phenols and the dilutions of the solutions. The maxima of each phenol appeared to obey the Lambert-Beer Law.

The effects of pH and of substrate concentration on the hydrolysis of the three nitrophenylsulphates are quoted in Table 2. In optimum conditions with these three substrates the extent of hydrolysis was proportional to time up to 2 hr. and to the quantity of takadiastase present up to 4% for the *o*- and *m*-substrates and up to 1.2% for the *p*-substrate.

The action of takadiastase on certain hydroxynitrophenylsulphates

The two ethereal sulphates, 4-hydroxy-2-nitro- and 4-hydroxy-3-nitro-phenylsulphuric acids (I and II) give rise to nitroquinol on hydrolysis. Nitroquinol on making alkaline gives a purple colour (λ_{max} 540 m μ ., ϵ_{max} 3850) which can be estimated with the Spekker absorptiometer. The colour

fades on standing, but is sufficiently stable to allow readings of its intensity to be taken. Similarly, 2-hydroxy-5-nitrophenylsulphuric acid (III) gives rise to 4-nitrocatechol which gives a relatively stable red colour in alkaline solution (λ_{max} 510 m μ ., ϵ_{max} 11 300) which again can be estimated in the colorimeter. The alkaline nitrocatechol colour is more intense than that of nitroquinol and is therefore more suitable for the assay of arylsulphatase. The standard curves for nitroquinol and nitrocatechol were constructed by dissolving 10-70 $\mu g.$ of nitroquinol or 4-30 $\mu g.$ of 4-nitrocatechol in 2 ml. water and adding 0.5 ml. of 2.5N-NaOH. The intensity of colour varied linearly with the amount of the two phenols. The nitroquinol and nitrocatechol colours were read immediately in the Spekker absorptiometer with

Ilford no. 604 (Spectrum green) filters, and using 1 cm. cells holding 0.5 ml. of solution.

Method. The substrate solution (1 ml.), made by dissolving the potassium salt of the ethereal sulphate in *m*-acetate buffer, was added to each of three tubes. To two of the tubes 1 ml. of the aqueous enzyme solution was added and all three tubes were incubated at 37° for the required time. To each tube there was then added 0.5 ml. of 2.5 *N*-NaOH and to the third tube (control) 1 ml. of enzyme solution. The colours of the two test solutions were then measured in the Spekker absorptiometer with the control solution in the blank cell of the instrument. The amount of nitroquinol or nitrocatechol liberated was then read from the standard curves.

Fig. 2. The effect of substrate concentration on the arylsulphatase activity of takadiastase. A, potassium 4-hydroxy-2-nitrophenylsulphate (I) as substrate, where no inhibition by excess substrate up to 0.005 *M* is seen; and B, potassium 2-hydroxy-5-nitrophenylsulphate (III) as substrate, showing inhibition by excess substrate.

The effect of pH and of substrate concentration on the hydrolysis of I and III by takadiastase is shown in Table 2. In the case of III there was an inhibition by excess of substrate which is shown graphically in Fig. 2.

In optimum conditions with I and III extent of hydrolysis was proportional to time up to 2 hr. and to the quantity of takadiastase present up to 4 %.

In the case of the 4-hydroxy-3-nitrophenylsulphate (II) incubation up to 4 hr. only released 6 µg. of nitroquinol, so that takadiastase appeared to have practically no hydrolytic action towards this ethereal sulphate. This sulphate was also more resistant to acid hydrolysis than its isomer (I), for I is completely hydrolysed by 0.25 *N*-H₂SO₄ at 98° in less than 10 min., whereas II requires at least 20 min. for complete hydrolysis under the same conditions.

Inhibition of arylsulphatase by 4-hydroxy-3-nitrophenylsulphate

Takadiastase shows very little hydrolytic activity towards potassium 4-hydroxy-3-nitrophenylsulphate (II). This could be due to the inability of the enzyme to combine with II to form the enzyme-substrate complex, or to the forma-

tion of a stable enzyme-substrate compound. If the second condition is correct then potassium 4-hydroxy-3-nitrophenylsulphate should behave as an inhibitor of arylsulphatase since it will combine under certain conditions with all the available enzyme and prevent its action on other hydrolysable substrates. The effect of potassium 4-hydroxy-3-nitrophenylsulphate upon the activity of takadiastase towards potassium 2-hydroxy-5-phenylsulphate was therefore studied.

Fig. 3. The inhibition by potassium 4-hydroxy-3-nitrophenylsulphate (II) of the hydrolysis of potassium 2-hydroxy-5-nitrophenylsulphate (III) by takadiastase. For conditions, see text.

The hydrolytic action of 0.5 ml. of 2% takadiastase upon 0.0025 *M*-potassium 2-hydroxy-5-nitrophenylsulphate was measured as above, in the presence of increasing concentrations of potassium 4-hydroxy-3-nitrophenylsulphate. The results (see Fig. 3) showed that the latter substance was highly inhibitory, 50% inhibition being caused by a concentration of about 1.4×10^{-4} *M*.

The nature of the inhibition. The activity of 2% takadiastase at increasing concentrations of potassium 2-hydroxy-5-nitrophenylsulphate in the absence and presence of 2.5×10^{-4} *M*-potassium 4-hydroxy-3-nitrophenylsulphate was measured at pH 5.8. The plot of $1/v$ against $1/S$ showed an increase in slope (see Fig. 4) in the presence of the inhibitor, whereas the ordinate intercept had the same value as in the absence of the inhibitor, thus indicating competitive inhibition. According to Lineweaver & Burke (1934) the increase in slope = $K_s I / K_i$, where I is the inhibitor concentration and K_i is the dissociation constant of the enzyme-inhibitor complex. In this particular experiment $K_s (=K_m)$ was 6.8×10^{-4} and K_i was calculated to be 2.4×10^{-6} *M*. Thus the affinity of potassium 4-hydroxy-3-nitrophenylsulphate for the enzyme was about 300 times ($6.8 \times 10^{-4} / 2.4 \times 10^{-6}$) that of potassium 2-hydroxy-5-nitrophenylsulphate.

*Inhibition of arylsulphatase activity
by some inorganic compounds*

It has been shown that arylsulphatase is inhibited by compounds which react with aldehyde groups, for KCN, K_2SO_3 , phenylhydrazine and NH_2OH are inhibitory (Soda, Egami, Koyama & Horigome, 1939; Rozenfeld & Ruchelman, 1940; Dziaszowski, 1950). Rozenfeld & Ruchelman (1940) concluded that arylsulphatase contained an aldehyde group in its prosthetic group. Furthermore, it has been reported that oxalate, fluoride, sulphate and phosphate ions and heavy metals inhibit the enzyme (Tanaka, 1938; Inouye, 1929; Dziaszowski, 1950; Huggins & Smith, 1947). According to Tanaka (1938) and Seligman, Chauncey & Nachlas (1951) $MgCl_2$ activates the enzyme, whereas Hommerberg (1931) claims that this compound inhibits slightly. Other workers have obtained activation of the enzyme by $Fe(OH)_3$, $BaCO_3$ and $CaCO_3$. These various workers, however, were working with arylsulphatases from different sources.

Fig. 4. Competitive inhibition of the hydrolysis of potassium 2-hydroxy-5-nitrophenylsulphate (III) by potassium 4-hydroxy-3-nitrophenylsulphate (II). A, in the presence of the inhibitor; B, in its absence (see text). v = rate of hydrolysis, expressed as $\mu g.$ 4-nitrocatechol/hr. S = substrate concn. (M).

Using potassium *p*-chloro- and *p*-nitro-phenylsulphates and takadiastase we have examined the action of a number of compounds on arylsulphatase.

Methods

(a) *Using p-chlorophenylsulphate.* The method described earlier (p. 203) was used, but the 0.4 ml. of substrate solution was replaced by 0.2 ml. inhibitor and 0.2 ml. substrate dissolved in 0.5 M-acetate buffer of pH 6.15. The final concentration of substrate was 0.0125 M. A similar change was made in the control tubes where 0.2 ml. each of inhibitor and substrate replaced the 0.4 ml. of substrate solution.

(b) *Using p-nitrophenylsulphate.* The inhibitor solutions were made up in M-acetate buffer and adjusted to pH 6.0

with acetic acid or 0.1 N-NaOH as necessary. The inhibitor solution (2 ml.), 0.01 M-substrate solution (2.5 ml.) and 2% takadiastase (0.5 ml.) were mixed in duplicate and incubated for 1.5 hr. at 37°. The control tubes were incubated in the same way using inhibitor and buffer solutions but omitting the enzyme solution until the end of the reaction. The tubes were then all treated with 0.5 ml. of 2N-NaOH and the liberated *p*-nitrophenol measured.

Table 3. *Effect of inhibitors on arylsulphatase activity of takadiastase*

Compound tested	Final concentration of inhibitor (M)	<i>p</i> -Chlorophenol liberated ($\mu g.$)	Inhibition (%)
<i>p</i> -Chlorophenylsulphate as substrate			
None	—	34.4	—
NaCl	0.02	34.7	0
Na_2SO_4	0.02	34.6	0
$MgCl_2$	0.02	34.5	0
Na_2HPO_4	0.02	27.9	19
Na_2SO_3	0.01	11.0	68
<i>p</i> -Nitrophenylsulphate as substrate			
None	—	20.0	—
KCl	0.02	20.0	0
K_2SO_4	0.02	20.0	0
$MgCl_2$	0.02	14.6	27
Na_2HPO_4	0.02	19.2	4
K_2SO_3	0.02	4.95	75
K_2SO_3	0.002	16.0	20
C_6H_5OH	0.02	18.0	10
NH_2OH, HCl	0.02	2.2	89
NH_2OH, HCl	0.002	7.7	61.5
KCN	0.02	0	100
KCN	0.002	10.7	46.5

Some of our results are given in Table 3. They confirm that compounds reacting with aldehyde groups (KCN, K_2SO_3 , Na_2SO_3 and NH_2OH) inhibit the enzyme. The effect of phosphates was slight but nevertheless evident. $MgCl_2$ (cf. Hommerberg, 1931) seemed to inhibit slightly when *p*-nitrophenylsulphate was used as substrate but not when *p*-chlorophenylsulphate was used.

DISCUSSION

The present work suggests that arylsulphatase can be estimated with almost any phenolic ethereal sulphate hydrolysed by sulphatase at a reasonable velocity provided that the anion of the liberated phenol absorbs light at a different wavelength from the ethereal sulphate. This probably occurs with most phenols. For the most sensitive methods, however, the extinction of the phenolic anion at its wavelength of maximum absorption must be large and of the phenols studied here the *p*-nitro-, *p*-chloro- and *p*-aldehyde-phenols and 4-nitrocatechol seem to satisfy these criteria (see Table 1). The use of *p*-nitrophenol and its sulphate for the assay of sulphatase has already been described (Huggins & Smith, 1947). The convenient maximum absorptions of the *p*-chloro- and *p*-aldehyde-phenol anions

occur in the ultraviolet and are therefore measured with a spectrophotometer covering the ultraviolet. With *p*-nitrophenol, both a colorimeter and a spectrophotometer can be used for measuring the yellow colour of its anion, the latter instrument being more convenient. Since 4-nitrocatechol gives a red colour in alkali, it is readily measured in a colorimeter. We have found the method using 4-nitrocatechol sulphate as substrate readily applicable to the assay of arylsulphatase in animal tissues, the colour being measured with a Spekker absorptiometer. This method is being further developed.

Takadiastase has been shown to hydrolyse the ethereal sulphates of phenol (Neuberg & Kurono, 1923), *p*-cresol (Neuberg & Lindhardt, 1923) *m*- and *p*-chloro-, *p*-bromo-, *o*-, *m*- and *p*-nitro-phenols, quinol, carvacrol, vanillin, *p*-hydroxybenzoic acid, indoxyl, 2-hydroxyquinoline (Neuberg & Wagner, 1925), salicylic acid (Morimoto, 1937), β -naphthol (Noguchi, 1924), oestrogens (Cohen & Bates, 1949), ($\pm$)-3-methylcyclohexylphenol (Weinmann, 1929) and (+)-*p*-sec.-butylphenol (Fromageot, 1929). It

and V, to give I and III respectively, causes the optimum pH for sulphatase activity to become more acid (see Table 4). Thus the optimum for IV is pH 6.3 moving to 6.0 in I, whilst the optimum for V is 6.3 moving to 5.9 in III. There is also in both cases a similar change in optimum substrate concentration which is 0.006 M for IV and V and about 0.002 M for I and III. Furthermore, K_m for IV and V is about 2.5 times the K_m for I and III ($K_{mIV}/K_{mI} = 2.7$ and $K_{mV}/K_{mIII} = 2.4$). Thus the introduction of a hydroxyl group into these sulphates increases the affinity of the enzyme for the substrate about 2.5 times.

On these grounds it seemed probable that the lack of hydrolytic activity of takadiastase towards II might be due to the formation of a stable enzyme-substrate complex. That this was actually the case was proved by the fact that II competitively inhibited the action of sulphatase on III. It was calculated that the affinity of the enzyme for II was about 280 times that for III and about 50 times that for I.

Table 4. Action of the arylsulphatase of takadiastase upon various ethereal sulphates

Ethereal sulphate	$K_m \times 10^4 M$	Optimum pH	Optimum substrate concentration (M)	Phenol liberated/hr. by		1 mg. of the N of the takadiastase preparation ($\mu g.$)
				1 g. of takadiastase		
				($\mu g.$)	($\mu mole$)	
<i>o</i> -Nitrophenyl	3.4	6.0-6.5	0.006	1100	7.8	275
<i>m</i> -Nitrophenyl	8.35	5.9-6.5	0.006	1750	12.5	437
<i>p</i> -Nitrophenyl	1.7	6.2	0.015	1272	9.15	318
4-Hydroxy-2-nitrophenyl	1.25	5.9-6.1	0.002	1426	9.2	356
2-Hydroxy-5-nitrophenyl	3.5	5.9	0.0025	837	5.4	209
4-Hydroxy-3-nitrophenyl	(0.024)*	—	—	0	0	—
<i>p</i> -Chlorophenyl	9.7	6.15	0.01	1610	12.5	402
<i>p</i> -Aldehydophenyl	2.1	5.5-6.1	0.0027	960	7.6	240

* K_I measured at pH 5.8.

failed to hydrolyse the ethereal sulphate of (–)-*p*-sec.-butylphenol (Fromageot, 1929). Our present results cover eight ethereal sulphates of which *o*-, *m*- and *p*-nitro- and *p*-chloro-phenylsulphates have been studied before. Amongst our results we have two examples of the effect on sulphatase activity of the introduction of a hydroxyl group into the aromatic ring of the nitrophenylsulphates. Thus we can compare 4-hydroxy-2-nitrophenylsulphate (I)

with *o*-nitrophenylsulphate (IV) and 2-hydroxy-5-nitrophenylsulphate (III) with *m*-nitrophenylsulphate (V). The introduction of an OH into IV

Our results also enable us to compare the action of sulphatase on three position isomers, namely the *o*-, *m*- and *p*-nitrophenylsulphates. The affinity ($1/K_m$) of the enzyme is greatest for the *p*- and least for the *m*-isomer, the ratio $1/K_{m,p} : 1/K_{m,o} : 1/K_{m,m}$ being 5:2.5:1.

Although $1/K_m$ is least for *m*-nitrophenylsulphate, more *m*-nitrophenol is liberated per hour by the enzyme from *m*-nitrophenylsulphate than *o*- and *p*-nitrophenols from their corresponding sulphates (Table 4).

The commercial preparation of takadiastase used in this work contained much added lactose and only a small amount of it was mould material. The nitrogen content was 0.4% and the protein content 2.5% (0.4×6.25). On the basis of its nitrogen content and the fact that most of the material of the preparation was added lactose, the original dried mould preparation must be a relatively good source of aryl-

sulphatase, for the enzyme in 1 g. of the takadiastase nitrogen would release 32 mg. of *p*-nitrophenol (see Table 4), for example, from the corresponding ethereal sulphate in 1 hr. at 37°.

Our experiments were carried out at 37°, and Abbott (1947) has reported that the optimum temperature of the enzyme is 50°. From Abbott's results arylsulphatase appeared to be about 50% more active at 50° than at 37°.

SUMMARY

1. A study has been made of the action of the arylsulphatase activity of takadiastase towards the alkali salts of *o*-, *m*- and *p*-nitro-, *p*-chloro-, *p*-aldehyde-, 4-hydroxy-2-nitro-, 4-hydroxy-3-nitro- and 2-hydroxy-5-nitro-phenylsulphuric acids.

2. The liberated phenols were determined spectrophotometrically or colorimetrically as their

anions, which have different spectra from the corresponding ethereal sulphates. The spectra of these sulphates are recorded.

3. Optimum pH's, Michaelis constants and optimum substrate concentrations have been determined for these ethereal sulphates.

4. Potassium 4-hydroxy-3-nitrophenylsulphate was not hydrolysed by takadiastase and inhibited the action of the enzyme towards other ethereal sulphates. This inhibition is competitive and is due to the formation of a stable enzyme-substrate complex.

5. A study has also been made of the inhibition of arylsulphatase by inorganic compounds and aldehyde reagents. It is confirmed that arylsulphatase is inhibited by compounds reacting with aldehydes.

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Seasonal Changes in the Carbohydrates of the Jerusalem Artichoke Tuber

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Several workers (Dubrunfaut, 1867; Colin, 1919; Thaysen, Bakes & Green, 1929; Dedonder, 1951c) have noted the change in carbohydrate composition which takes place when the tubers of the Jerusalem artichoke (*Helianthus tuberosus* L.) are stored over the winter. This change may be inferred from the optical rotation of the expressed juice, which changes in the positive direction, and is confirmed

by the observations that in the spring less inulin may be obtained from the tubers and more of the carbohydrate is fermentable. Similar observations have been made on chicory, *Cichorium Intybus* L., by Wolff & Geslin (1918).

Bacon & Edelman (1951) suggested that all these changes could be the result of a redistribution of fructose residues among the many carbohydrate