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Studies on Sulphatases

1. THE CHOICE OF SUBSTRATE FOR THE ASSAY OF RAT-LIVER ARYLSULPHATASE

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The existence of arylsulphatase was first demonstrated by a colorimetric procedure when Derrien (1911), using an extract of the gastropod *Murex trunculus*, liberated indoxyl from indoxylsulphate. The colour of the indigo formed by subsequent oxidation of the indoxyl led Derrien to name the enzyme purpurase. No attempt was made to place this observation on a quantitative basis and the first measurements of arylsulphatase activity were confined to the estimation of the liberated sulphate ion by gravimetric (Neuberg & Kurono, 1923) and turbidimetric methods and potentiometric titration (Soda, 1936). Although these methods possess the inherent advantage of applicability to the assay of all sulphatases, using any of the appropriate sulphate esters, they are limited in practice by their low sensitivity. Incubation periods of up to 20 days were sometimes necessary. The benzidine (Tanaka, 1938*a, b*) and manometric methods (Lindahl, 1935) of sulphate estimation, as adapted to sulphatase assay, are more sensitive, but the conditions for assay under varying circumstances have not been fully established.

Attention has thus been turned towards estimation of the phenols liberated from arylsulphates, and for this purpose several colorimetric and spectrophotometric methods are available. Briefly the methods are of two types: (i) using phenols which, in the anionic form, absorb light maximally at wavelengths in the visible or ultraviolet regions of the spectrum where the corresponding ethereal sulphate has negligible absorption; (ii) using phenols which can be estimated colorimetrically after a chemical reaction which does not affect the respective arylsulphate.

A colorimetric method of type (i) was introduced by Morimoto (1937) who used a comparator to measure the yellow *p*-nitrophenol released from *p*-nitrophenylsulphate. Huggins & Smith (1947) modified the method for the Evelyn photoelectric colorimeter and later greater sensitivity was achieved for the estimation of the isomeric mononitrophenols by the use of the spectrophotometer (Robinson, Smith, Spencer & Williams, 1952). A similar colorimetric method has been developed by Robinson, Smith & Williams (1951*a*), using the red

colour of the 4-nitrocatechol anion liberated from 2-hydroxy-4-nitrophenylsulphate. In the ultra-violet range the sulphates of *p*-chlorophenol and *p*-hydroxybenzaldehyde have been used as substrates for the measurement of the arylsulphatase activity of takadiastase, the liberated phenols being estimated spectrophotometrically (Robinson *et al.* 1952).

Two methods of type (ii) have been described. Phenol released from phenylsulphate was determined after colour formation with Folin-Ciocalteu reagent (Abbott, 1947); 6-bromo-2-naphthol from its sulphate ester was coupled with tetrazotized *o*-dianisidine and the resultant dye estimated after extraction with ethyl acetate (Seligman, Chauncey & Nachlas, 1951). Robinson, Smith & Williams (1951*b*) have used the same coupling reagent with 8-hydroxyquinoline for the assay of β -glucuronidase and have suggested the similar use of 8-hydroxyquinoline as a substrate for arylsulphatase. No details have been presented.

Assay of the arylsulphatase of rat tissues has been confined to the *p*-nitrophenol (Huggins & Smith, 1947; Abbott & East, 1949), and 4-nitrocatechol methods. During repetition of this work it became apparent that the arylsulphatase of rat liver was not fully released in extracts prepared according to the direction of Huggins & Smith (1947) and Robinson *et al.* (1951*a*). Such extracts contained only a proportion of the total arylsulphatase of the liver tissue and large amounts remained in the extracted material (see Dodgson, Spencer & Thomas, 1953). Whole homogenates were therefore used, but recovery of added *p*-nitrophenol and 4-nitrocatechol from such homogenates was low. Huggins & Smith make no record of recovery experiments, and the successful recovery of 4-nitrocatechol has been attempted with tissue extracts only (R. T. Williams, private communication).

Recovery experiments of phenols used in the available arylsulphatase assay methods from whole homogenates of rat tissues have been carried out, and a search made for other phenols which can be quantitatively recovered and which are suitable for the estimation of arylsulphatase.

The colour reaction for the estimation of phenol (Abbott, 1947) is relatively insensitive and unspecific, and the procedure is laborious in comparison with other methods. The absorption maxima of *p*-chlorophenol are low and occur at wavelengths where protein absorbs strongly. (Spencer & Williams, 1950). For these reasons no attempt has been made to study the recovery of phenol or *p*-chlorophenol from rat tissues.

EXPERIMENTAL AND RESULTS

Animals and materials. Rats of the Medical Research Council hooded strain were used throughout. Occasional

albinos were produced in some litters. Commercially available phenols were recrystallized and melting points taken as criteria of purity. The 4-nitrocatechol (m.p. 173–175°) was prepared by the method of Benedikt (1878) with the introduction of a steam-distillation stage to remove 3-nitrocatechol and unchanged catechol. The *p*-hydroxybenzyl alcohol (m.p. 125°) was prepared according to Auwers & Daecke (1899) and 6-bromo-2-naphthol (m.p. 129–130°) according to Koelsch (1940). The isolation of *p*-hydroxyhippuric acid from human urine followed the method of Quick (1932). All melting points are uncorrected.

Preparation of potassium p-acetylphenylsulphate. The method of Burkhardt & Lapworth (1926) was modified. Diethylaniline (40 g., 43 ml.) was dissolved in 40 ml. CS₂ and cooled in an ice-salt mixture. Chlorosulphonic acid (13.6 g., 17.7 ml.) was added dropwise to the mixture with stirring so that the temperature did not rise above 5°. After the addition the temperature was allowed to rise to that of the room and 14.5 g. *p*-hydroxyacetophenone were added as a suspension in CS₂. The mixture was stirred for an hour and allowed to stand a further hour or overnight as was convenient. After removal of the lower layer of CS₂ and excess diethylaniline, the brown syrup of the diethylaniline salt of *p*-acetylphenylsulphuric acid was poured slowly with stirring into 30 ml. water containing 14.5 g. KOH and 4 g. Ba(OH)₂. Care was taken to ensure that the temperature did not rise above 35° and that the final mixture was alkaline. The sludge was filtered by suction until dry. The dry material was suspended in 250 ml. ethanol, filtered and washed well with ethanol followed by ether. Extraction of the potassium *p*-acetylphenylsulphate was achieved by boiling the washed and dried material with 35 ml. water for a few seconds and filtering after treatment with charcoal. The filtrate deposited needle crystals of the arylsulphate which were separated, dried by suction and washed well with ethanol and ether to remove contaminating CS₂ and diethylaniline. Recrystallization from small volumes of water was repeated (usually two or three times) until the compound was free of inorganic sulphate. A further recrystallization was made from aqueous ethanol. Analysis of the compound gave C, 37.3; H, 2.8; S, 12.1; K, 14.3. C₈H₇O₆SK requires C, 37.7; H, 2.8; S, 12.6; K, 15.4%. The compound was free from Cl[−] and SO₄^{2−} but contained small traces of the parent phenol (ϵ_{max} , 160). This state of purity is sufficient for most enzyme studies. Four further recrystallizations from aqueous ethanol gave a product apparently free from *p*-hydroxyacetophenone since the molecular extinction coefficient at 323 m μ . in 0.1 N-NaOH did not decrease on further recrystallization.

Spectrophotometric estimation of phenols added to tissue homogenates

A standard procedure, based on that of Spencer & Williams (1951) for *p*-chlorophenol, was used for the estimation of *p*-nitrophenol, *p*-hydroxybenzaldehyde, 4-nitrocatechol and *p*-hydroxyacetophenone. Tissue homogenate, buffer and phenol were incubated together, 'deproteinized' with ethanol (final concentration 80%) and alkali added to convert the phenol into the anionic form. The phenol was then measured spectrophotometrically at the wavelength of its maximum absorption. Controls were prepared in a similar manner but omitting the phenol.

Table 1. *Absorption spectra of several phenols*

Phenol	Solvent					
	0.1 N-NaOH		66.7% Ethanolic NaOH (0.167 N)		0.1 N-HCl	
	$\lambda_{\max.}$ (m μ .)	$\epsilon_{\max.}$ (m μ .)	$\lambda_{\max.}$ (m μ .)	$\epsilon_{\max.}$ (m μ .)	$\lambda_{\max.}$ (m μ .)	$\epsilon_{\max.}$ (m μ .)
<i>p</i> -Nitrophenol	227.5	5 850	228	6 200	226	7 100
	400.0	18 200	402.5	21 000	315.5	9 900
4-Nitrocatechol*	510	11 200	510	11 200	—	—
<i>p</i> -Hydroxybenzaldehyde	237.5	7 680	241	7 350	218	11 600
	330	27 500	335	30 200	285	16 000
<i>p</i> -Hydroxyacetophenone	235	5 700	238	6 000	218.5	8 600
	323	20 500	327.5	21 700	274	12 450

* Measured in the visible range only.

Table 2. *Conditions for the measurement of recovered phenols*(For definition of *F* see text.)

Phenol	Light source	Slit width (mm.)	Wavelength (m μ .)	<i>F</i>
<i>p</i> -Nitrophenol	Filament	0.3	402.5	46.33
4-Nitrocatechol	Filament	0.16	510	96.81
<i>p</i> -Hydroxybenzaldehyde	Hydrogen	0.5	335	28.28
<i>p</i> -Hydroxyacetophenone	Hydrogen	0.5	327.5	43.86

The final solution contained approximately 66.6% (v/v) ethanol and was 0.167 N with respect to sodium hydroxide. The absorption spectra of some phenols under these conditions differ slightly from those observed in aqueous sodium hydroxide. The data are presented in Table 1. The spectra of the unchanged form of the phenols are included since they approximate closely to those of the corresponding *O*-conjugate in both hydrochloric acid and sodium hydroxide (see Discussion). All absorption spectra were determined with a Hilger 'Uvispek' spectrophotometer and the quartz prism. Quartz cells, 1 cm. optical length, were used throughout.

Estimation procedure. The fresh rat tissue was homogenized in 0.5 M-acetate buffer at the required pH with a glass homogenizer (Potter & Elvehjem, 1936) or a macerator similar in design to that of Campbell & Davidson (1949). The method of tissue preparation did not appear to influence the recoveries of the phenols. The results reported below are those obtained with homogenates prepared by grinding the freshly removed wet tissue with the buffer for 2 min. in a glass homogenizer under ice-cold conditions.

The homogenate (0.6 ml.) was added to each of four 15 ml. centrifuge tubes which were then placed in a water bath at 37.5°. After 4 min. the phenol, dissolved in 0.6 ml. 0.5 M-acetate buffer at the same pH as the homogenate, was added to two of the tubes (test) and 0.6 ml. of the buffer without the phenol added to the remaining two tubes (control). The solutions were mixed and the tubes stoppered and incubated for the requisite period. After the addition of 4.8 ml. ethanol at the end of the incubation period the tubes were sealed with rubber caps and centrifuged to remove

protein. A 5 ml. portion of the clear supernatant of each tube was added to 1 ml. N-NaOH and the absorption at the wavelength of maximum absorption (see Table 2) measured against an ethanol blank. In the case of 4-nitrocatechol the period between the addition of ethanol and the measurement of the absorption was less than 10 min., since the colour faded after this time due to atmospheric oxidation.

The amount of phenol present in the incubation mixture is given by

$$\mu\text{g. phenol} = \frac{(E_t - E_c) \times \text{mol. wt.} \times 5.88 \times 5.94 \times 10^6}{\epsilon_{\max.} \times 5 \times 10^3},$$

or

$$\mu\text{g. phenol} = (E_t - E_c) \times F,$$

E_t and E_c are the observed extinctions of test and control solutions and $\epsilon_{\max.}$ the molar extinction coefficient in 66.6% (v/v) ethanolic NaOH (0.166 N) (Table 1). Values of *F* are given in Table 2. The numbers 5.88 and 5.94 occurring in the equation differ from 6.00 because of the shrinkages of volume which occur on mixture of 4.8 ml. ethanol and 1.2 ml. incubation mixture, and on mixture of 5 ml. of this solution with 1 ml. N-NaOH. At the wavelengths of maximum absorption of the respective phenols the Lambert-Beer Law appeared to be obeyed in the range of log I_0/I 0.050–1.500 for the four phenols under the conditions of the experiment.

Estimation of 6-bromo-2-naphthol. The improved method using CHCl_3 for extraction of the dye was employed (Cohen, Tsou, Rutenburg & Seligman, 1952). Stabilized tetrazotized *o*-dianisidine is available in this country under the trade name of Brentamine Fast Blue B Salt (Imperial Chemical Industries Ltd.). The incubation mixture was composed of 0.6 ml. homogenate in acetate buffer and 5 ml. water containing the 6-bromo-2-naphthol. Controls without 6-bromo-2-naphthol were run simultaneously.

Recovery experiments

Recoveries were tested from pure solution, and from solutions containing up to 2 g. wet wt. tissue/100 ml. homogenate with and without incubation. Recovery from pure solution was in all cases $100 \pm 1\%$. From unincubated solution of homogenate, recoveries were similarly complete, except in the case of 6-bromo-2-naphthol: here only 70–80% was recovered, owing to absorption of the coupled dye on the trichloroacetic precipitate (cf. Cohen *et al.* 1952). Incubation did not further reduce the recovery of 6-bromo-2-naphthol and recoveries of the other phenols after incubation were $100 \pm 2\%$, except where liver tissue was used: here lower recoveries were obtained depending on the time of incubation (Table 3).

Recoveries of *p*-nitrophenol, 4-nitrocatechol and 6-bromo-2-naphthol, were checked at the pH used in the original assays of rat tissue arylsulphatase (Abbott & East, 1949; Robinson *et al.* 1951a; Seligman *et al.* 1951). In the cases of *p*-hydroxybenzaldehyde and *p*-hydroxyacetophenone the optimum pH of rat arylsulphatase acting on the sulphate esters of these phenols was not known and recoveries were made in the pH range 6.0–7.2. Later work (Dodgson *et al.* 1953) has shown that the

optimum pH of rat liver arylsulphatase acting on potassium *p*-acetylphenylsulphate in acetate buffer is 7.2.

Explanation of low recoveries of p-nitrophenol and p-hydroxybenzaldehyde

Spectroscopic examination of incubation solutions after deproteinization. An examination of the spectrum of a *p*-nitrophenol 'test solution', prepared in the manner detailed in the estimation procedure, gave an indication of the fate of the *p*-nitrophenol lost after incubation with rat-liver homogenates. The spectrum in Fig. 1 is that of 37.9 μ g. *p*-nitrophenol after incubation with a 3.5% rat-liver homogenate for 2 hr. at pH 6.0 measured against a 'control solution' in the blank cell. There was a 16.9% loss of *p*-nitrophenol. The spectrum shows a small peak at 290–295 $m\mu$, which is absent from the spectrum of the *p*-nitrophenol anion in pure solution and after recovery from rat-liver homogenates without incubation. A possible metabolite, *p*-aminophenol, has an anionic absorption maximum in ethanol at 287 $m\mu$. (Morton & McGookin, 1934).

A similar spectroscopic examination was made of 22.8 μ g. *p*-hydroxybenzaldehyde after a 2 hr. incubation with a 2% liver homogenate at pH 6.6

Table 3. *Recovery of phenols from rat-tissue homogenates after incubation*

Tissue	Time of incubation (hr.)	Tissue in incubated mixture (%)	pH of incubated mixture	Phenol added (μ g.)	Recovery (%)
<i>p</i> -Nitrophenol					
Liver	1	2.0	6.6	19.6	88.0
Liver	2	2.0	6.6	19.6	80.7
Intestine	2	2.0	6.6	37.9	100.8
Kidney	2	1.5	6.6	37.9	99.4
Spleen	2	1.5	6.6	37.9	101.3
Muscle	2	2.0	6.6	37.9	100.5
4-Nitrocatechol					
Liver	1	2.1	6.6	30.0	90.2
Liver	2	2.1	6.6	30.0	87.7
<i>p</i> -Hydroxybenzaldehyde					
Liver	2	2.2	7.2	18.3	41.0
Liver	2	2.0	6.6	22.0	58.9
Liver	1.5	1.5	6.0	21.7	90.8
Kidney	1.5	1.5	6.0	21.7	98.2
Spleen	1.5	1.5	6.0	21.7	99.2
Muscle	1.5	2.0	6.0	21.7	102.4
<i>p</i> -Hydroxyacetophenone					
Liver	1	2.2	7.2	19.6	100.0
Liver	3	2.1	7.2	28.9	99.6
Liver	2	2.0	6.6	37.1	98.3
Liver	2	3.5	6.0	37.1	100.2
Kidney	3	2.4	7.2	28.9	98.6
Spleen	3	2.0	7.2	28.9	100.7
Brain	3	3.0	7.2	28.9	98.3
Intestine	3	2.5	6.0	37.1	100.7

resulting in a 46.1% loss. The spectrum showed a small flat peak in the region 275–280 $m\mu$. which is absent from the spectrum of pure *p*-hydroxybenzaldehyde (Fig. 1). Possible metabolites of *p*-hydroxybenzaldehyde which absorb in this region are *p*-hydroxybenzoic acid, its conjugates and *p*-hydroxybenzyl alcohol (see Table 5).

Fig. 1. Absorption spectra of solutions of *p*-hydroxybenzaldehyde (—) and *p*-nitrophenol (---) after incubation with rat-liver homogenates. Incubation mixtures were deproteinized with ethanol and then made alkaline with NaOH.

Paper chromatography. Extracts of solutions of *p*-nitrophenol and *p*-hydroxybenzaldehyde after incubation with liver homogenates were examined chromatographically for the presence of metabolites.

A 10% homogenate of rat liver in 0.5M-acetate buffer, pH 6.6 (10 ml.), was incubated with four 5 ml. portions of buffer, two of which contained *p*-nitrophenol (324 $\mu g.$) and *p*-hydroxybenzaldehyde (107 $\mu g.$) respectively. After 3 hr. the *p*-nitrophenol mixture and one of the control tubes were adjusted to pH 7.5 with M-acetate whilst the *p*-hydroxybenzaldehyde mixture and the other control were made acid to Congo red with a few drops of N-HCl. All four solutions were extracted with 25 ml. ether, centrifuged to disperse the emulsion and the ether layer separated, dried and con-

centrated. Quantitative experiments carried out simultaneously indicated that there was a 46% loss of *p*-nitrophenol and an 83% loss of *p*-hydroxybenzaldehyde.

The *p*-nitrophenol-homogenate extract and reference compounds were chromatographed using wet isopropyl ether and Barcham-Green no. 401 paper (Robinson, Smith & Williams, 1951c). After equilibration overnight and a 3 hr. run the dried chromatogram was sprayed with a mixture of 5 ml. N-HCl and 2.5 ml. 5% (w/v) NaNO₂ followed by 5% (w/v) thymol in 20% (w/v) Na₂CO₃. The presence of both *p*-nitrophenol and *p*-aminophenol in the extract was established; the former appearing as a yellow spot at the solvent front and the latter as a blue spot (R_f 0.60). The control homogenate extract gave no spots.

The *p*-hydroxybenzaldehyde-homogenate extract was chromatographed on Whatman no. 1 paper along with reference spots of *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, *p*-hydroxybenzyl alcohol and *p*-hydroxyhippuric acid. After equilibration overnight the paper was run for 3 hr. with the non-aqueous phase of benzene:acetic acid:water (40:40:20). The spots were developed with alkaline KMnO₄ or the diazotized *p*-nitroaniline reagent of Bray, Thorpe & White (1950).

The results of the experiment are given in Table 4 and indicate that *p*-hydroxybenzaldehyde is oxidized in part to *p*-hydroxybenzoic acid and reduced to *p*-hydroxybenzyl alcohol. No *p*-hydroxyhippuric acid was detected. Confirmatory evidence of the composition of the spots from the homogenate extract was obtained spectrophotometrically. The spots were eluted from the paper with 0.1N-sodium hydroxide and examined against a 0.1N-sodium hydroxide blank. A portion of the eluate was made 0.1N with respect to hydrochloric acid and spectrophotometrically examined against a suitable blank. The results shown in Table 5 confirm the identity of the metabolites of *p*-hydroxybenzaldehyde as the corresponding acid and alcohol. No *p*-hydroxybenzyl alcohol could be detected in chromatograms of extracts of *p*-hydroxybenzoic acid after incubation with liver homogenates.

Quantitative estimation of *p*-aminophenol. No metabolite other than *p*-aminophenol was detected after incubation of *p*-nitrophenol with rat-liver homogenate. It was of interest, therefore, to

Table 4. R_f values of *p*-hydroxybenzaldehyde and related phenols

(For conditions see text.)

Phenol	R_f	Colour with alkaline KMnO ₄	Colour with diazotized <i>p</i> -nitroaniline
<i>p</i> -Hydroxybenzaldehyde	0.370	Yellow	None
<i>p</i> -Hydroxybenzoic acid	0.330	Yellow	Red
<i>p</i> -Hydroxybenzyl alcohol	0.150	Yellow	Red
<i>p</i> -Hydroxyhippuric acid	0.000	Yellow	Red (faint)
Extract of <i>p</i> -hydroxybenzaldehyde after incubation with rat-liver homogenate	0.370	Yellow	None
	0.330	Yellow	Red
	0.150	Yellow	Red
Homogenate control	None	None	None

Table 5. Comparison of the spectra of spots eluted from paper chromatograms with those of *p*-hydroxybenzyl alcohol and *p*-hydroxybenzoic acid

	Solvent			
	0.1N-NaOH		0.1N-HCl	
Phenol	$\lambda_{\max}$ (m μ .)	$\epsilon_{\max}$ (m μ .)	$\lambda_{\max}$ (m μ .)	$\epsilon_{\max}$ (m μ .)
Spot R_F 0.150	243	—	222	—
	290	—	272	—
* <i>p</i> -Hydroxybenzyl alcohol	243	11 820	223	7 950
	290	2 590	273	1 360
Spot R_F 0.330	280	—	255	—
<i>p</i> -Hydroxybenzoic acid	281	13 400	255	14 000

* Moir (1923) gives $\lambda_{\max}$ 293 m μ .

establish the proportion of the loss of *p*-nitrophenol that could be accounted for by the reduction.

Acetate buffer (2.5 ml., 0.5M, pH 6.6) containing 152 μ g. *p*-nitrophenol was incubated for 2 hr. with 5 ml. of a 6% rat-liver homogenate in the same buffer. The incubation mixture was then centrifuged. A portion of the supernatant (1.2 ml.) was deproteinized with 4.8 ml. ethanol and the *p*-nitrophenol content estimated spectroscopically. Another portion (5 ml.) was assayed for *p*-aminophenol by the indophenol method detailed by Robinson *et al.* (1951c) using 8 ml. $K_3Fe(CN)_6$.

Suitable control determinations were made. The results showed that there was a 44 μ g. loss of *p*-nitrophenol of which 42.1 μ g. could be accounted for as *p*-aminophenol. Further experiments confirmed that *p*-nitrophenol was quantitatively reduced to *p*-aminophenol (94–100%, five determinations).

Potassium *p*-acetylphenylsulphate

Recoveries of *p*-hydroxyacetophenone from various rat-tissue homogenates were complete, and the phenol method seemed applicable to arylsulphatase assay. It remained to be established that the absorption of potassium *p*-acetylphenylsulphate was sufficiently low at the wavelength of maximum absorption of *p*-hydroxyacetophenone (323 m μ . in sodium hydroxide, 327 m μ . in ethanolic sodium hydroxide) to allow small quantities of the phenol to be estimated in the presence of relatively large amounts of the sulphate. In 0.1N-sodium hydroxide, potassium *p*-acetylphenylsulphate has $\lambda_{\max}$ 252.5 m μ ., $\epsilon_{\max}$ = 11 450, whilst at 323 m μ . ϵ_{323} = 70 (Fig. 2). In a solution in 0.167N-sodium hydroxide containing 66.7% (v/v) ethanol, the sulphate had $\epsilon_{327.5}$ = 75. These values indicate that the sulphate of *p*-hydroxyacetophenone may be of use as a substrate for arylsulphatase assay.

DISCUSSION

The unsuitability of various substrates for rat-liver arylsulphatase. Various colorimetric and spectrophotometric methods of arylsulphatase assay have

been reported. They are based on the estimation of phenols liberated from the corresponding ethereal sulphates after incubation with the source of the enzyme, and it is essential in such methods that the recoveries of the phenols from the enzyme extracts should be quantitative. In the study of rat arylsulphatase whole homogenates of the tissues must

Fig. 2. Absorption spectra of *p*-hydroxyacetophenone (—) and potassium *p*-acetylphenylsulphate (---) in 0.1N-NaOH and *p*-hydroxyacetophenone in 0.1N-HCl (.....).

be used in order to obtain the full activity of the enzyme. The present work shows that recovery of *p*-nitrophenol, *p*-hydroxybenzaldehyde, 4-nitrocatechol and 6-bromo-2-naphthol from whole homogenates of rat liver is incomplete and the methods of assay using these phenols are therefore unsuitable in these circumstances.

The evidence presented here suggests that the low recovery of *p*-nitrophenol can be accounted for by the formation of *p*-aminophenol. It is possible that the similar loss of 4-nitrocatechol may also be attributed to the reduction of the nitro group since the reduction of nitro groups by tissue preparations *in vitro* appears to be a reaction common to most

aromatic nitro compounds. Parker (1952) has followed the reduction of 2:4-dinitrophenol and the isomeric aminonitrophenols by rat-liver homogenates and has investigated the reaction more fully.

The action of rat liver on *p*-hydroxybenzaldehyde *in vitro* results in the formation of *p*-hydroxybenzoic acid and *p*-hydroxybenzyl alcohol. The production of these metabolites is easily explained by the action of aldehyde mutase, but Racker (1949) has produced evidence which suggests that the mutase activity is due to two separable enzymes, aldehyde oxidase and aldehyde reductase (alcohol dehydrogenase), both of which are present in liver. Aldehyde oxidase, although not able to oxidize benzaldehyde, shows some activity towards *o*-hydroxybenzaldehyde (Racker, 1949), and it is possible that it attacks the para isomer as well. The formation of *p*-hydroxybenzoic acid could also be brought about by xanthine oxidase, but no attempt has been made here to determine the enzyme responsible for the oxidation. Alcohol dehydrogenase has been reported to attack aromatic alcohols at an insignificant rate and the reverse reaction, the reduction of aldehyde to alcohol, does not appear to have been studied in the aromatic series. Furthermore, after feeding aromatic aldehydes to animals only oxidation products of the ingested compounds have been isolated from the urine (see Williams, 1947). The present study shows, however, that *p*-hydroxybenzaldehyde, but not *p*-hydroxybenzoic acid, is considerably reduced by rat-liver homogenates. If this activity is attributed to aldehyde reductase the present limits of specificity of this enzyme must be widened to include the aromatic aldehydes. Although large percentages of the *p*-hydroxybenzaldehyde incubated with liver homogenates were oxidized to *p*-hydroxybenzoic acid the formation of *p*-hydroxyhippuric acid was not observed (cf. Cohen & McGilvery, 1948).

The choice of suitable substrate. A search has been made for phenols which can be recovered quantitatively from rat-tissue homogenates and which are suitable for arylsulphatase assay. It was considered that the estimation of a phenol as its anion by physical means was to be preferred to methods involving chemical reaction. The selection of a suitable phenol was assisted by the observation of certain empirical relationships between the structure of phenols, their *O*-conjugates and their spectra. The absorption maxima of phenols move to longer wavelengths on changing the ionic state by addition of alkali. Thus the phenol itself has λ_{max} 210.5 and 270 $\text{m}\mu$., whilst the anion absorbs maximally at 235 and 287 $\text{m}\mu$. (Doub & Vandenberg, 1947). The spectra of para-substituted phenols with substituent groups which possess neither a considerable characteristic absorption nor form a

unique chromophore with the aromatic ring are similar to those of phenol. An *O*-conjugate of the phenol may be regarded as a suitable substrate for the assay of the corresponding hydrolase if its absorption is sufficiently low at the wavelength of maximum absorption of the phenol anion to permit the estimation of small quantities of the phenol in the presence of large amounts of its conjugate. An indication of the suitability of the *O*-conjugate may be obtained by examination of the spectrum of the corresponding phenol. If the compound which forms the *O*-conjugate of the phenol does not possess a characteristic absorption the spectrum of the conjugate in acid or alkali will approximate closely to that of the corresponding phenolic cation. Such a correlation exists for *p*-chlorophenol and its glucuronide (Spencer & Williams, 1951) and sulphate (Robinson *et al.* 1952). The glucosides, glucuronides, aliphatic esters, phosphates and sulphates of phenols exhibit this behaviour.

Monosubstituted phenols whose substituent groups make no major contribution to the spectra seldom have anionic maxima above 300 $\text{m}\mu$. and their measurement is thus susceptible to interference by protein which absorbs strongly below this wavelength (cf. Goodwin & Morton, 1946). Convenient deproteinization procedures, especially with crude homogenates, are often incomplete and fail to reduce protein absorption to a satisfactory level. The use of phenols having maxima above 310 $\text{m}\mu$. avoids the necessity for deproteinization, and protein precipitation need only be used for clarification of homogenates when necessary. Monosubstituted phenols with anionic maxima above 310 $\text{m}\mu$. are those in which the substituent group considerably influences the normal spectrum of phenol. Two such types of groups are (i) nitro group, (ii) a group containing a double bond which can exist with those of the aromatic ring as a conjugated double bond system, e.g.

Thus *p*-nitrophenol has λ_{max} 405 $\text{m}\mu$. in alkali and the anionic form of *p*-hydroxybenzaldehyde has λ_{max} 330. The similarity between the spectra of phenols of the two types mentioned above and those of their *O*-conjugates decreases as the substituent groups of the phenols contribute more to the spectra.

Unfortunately, the nitro and aldehydic groups are easily attacked by animal tissues *in vitro* and are therefore unsuitable chromogens. A rapid survey of the fate of phenols, which the general considerations above have suggested as suitable for the assay of hydrolases, showed that phenolic ketones appear to be resistant to attack other than

conjugation by the animal body. This class of compounds is excreted unchanged or conjugated as the glucuronide or ethereal sulphate whilst the ketone group remains intact (see Williams, 1947). The isomeric monohydroxyacetophenones were therefore considered. The para isomer was selected for study since its anionic maximum at 323 m μ . has a greater molecular extinction coefficient than those of the ortho and meta compounds at 349 and 359 m μ . (Doub & Vandenbelt, 1949). Since the results reported in this paper show that rat-tissue homogenates do not attack *p*-hydroxyacetophenone, and since potassium *p*-acetylphenylsulphate has very low absorption at 323 m μ ., the latter is a suitable substrate for arylsulphatase assay. Other *O*-conjugates of *p*-hydroxyacetophenone may be suitable substrates for the assay of the corresponding hydrolases.

SUMMARY

1. Recovery of *p*-nitrophenol, 4-nitrocatechol and *p*-hydroxybenzaldehyde was complete from all rat tissues tested except liver. Absorption by protein of coupled 6-bromo-2-naphthol accounted for low recoveries of this phenol.

2. Rat-liver homogenates reduced *p*-nitrophenol to *p*-aminophenol whilst *p*-hydroxybenzaldehyde was metabolized to *p*-hydroxybenzyl alcohol and *p*-hydroxybenzoic acid.

3. Rat-tissue homogenates including liver did not appear to metabolize *p*-hydroxyacetophenone.

4. Potassium *p*-acetylphenylsulphate is suggested as a suitable substrate for a spectrophotometric method of arylsulphatase assay.

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