The Specific Assay of Arylsulphatase C, a Rat Liver Microsomal Marker Enzyme

By D. W. MILSOM, F. A. ROSE and K. S. DODGSON
Department of Biochemistry, University College, P.O. Box 78, Cardiff CF1 1XL, U.K.

(Received 2 November 1971)

Conditions based on previous assays with potassium \( p \)-acetylphenyl sulphate have been established for the specific assay of arylsulphatase C in rat tissues. The enzyme has optimum activity with 40 mM substrate at pH 8.0 in the presence of 0.1 M-phosphate buffer. Under these conditions arylsulphatase C can be assayed without interference from the other arylsulphatase enzymes present and is useful as a marker for the endoplasmic reticulum in cell-fractionation studies.

Mammalian tissues appear to possess at least three arylsulphatase enzymes (aryl sulphate sulphohydrolases, EC 3.1.6.1) distinguishable by their activity towards various substrates, their tissue distribution and their intracellular localization (Dodgson et al., 1955). In rat liver two of these enzymes, arylsulphatases A and B, are localized mainly in the lysosomal fraction, whereas the third one, arylsulphatase C, is in the microsomal fraction where it is present in a highly insoluble form (Dodgson et al., 1954, 1957). The combined activity of arylsulphatases A and B measured under arbitrary conditions is frequently used in cell-fractionation studies as a marker for the lysosome fraction, and arylsulphatase C has been shown to be a valuable marker for the membrane component of the microsomal fraction (Spencer et al., 1955; Dodgson et al., 1957; Milsom et al., 1968; Milsom & Rose, 1970).

The commonly used assay methods for these enzymes are not entirely satisfactory, since they are largely dependent on relative differences in the activity of the enzymes towards various synthetic chromogenic aryl sulphates (Dodgson & Spencer, 1957) and some degree of cross-specificity between substrates is exhibited. Dipotassium 2-hydroxy-5-nitrophenyl sulphate (4-nitrocatechol sulphate) is commonly used for the assay of arylsulphatases A and B, since these enzymes have a high affinity for the compound. However, under the conditions of assay, although the affinity of arylsulphatase C for this substrate is appreciably lower it is not insignificant. Similarly, potassium \( p \)-nitrophenyl sulphate has frequently been used as a chromogenic substrate for the assay of arylsulphatase C (see Dodgson et al., 1955), but this substrate is also hydrolysed to some extent by the A and B enzymes. Moreover, it is unsuitable for use in fractionation studies on liver, since the liberated \( p \)-nitrophenol is not quantitatively recoverable from whole homogenates of this tissue. Potassium \( p \)-acetylphenyl sulphate, the substrate chosen by Dodgson et al. (1953) for measuring arylsulphatase C activity in rat tissues, has some advantage over potassium \( p \)-nitrophenyl sulphate in that the liberated \( p \)-hydroxyacetophenone is completely recoverable from whole homogenates. It has now been shown that potassium \( p \)-acetylphenyl sulphate can be used for the specific assay of arylsulphatase C, provided that the reaction is performed in the presence of a concentration of phosphate ions that is sufficient to inhibit arylsulphatases A and B completely.

Materials and Methods

Materials

Analytical-grade chemicals supplied by BDH Chemicals Ltd., Poole, Dorset, U.K., were used throughout.

Potassium \( p \)-acetylphenyl sulphate. The method of preparation described by Dodgson & Spencer (1953) was modified as follows. Diethylaniline (43 ml) was dissolved in carbon disulphide (40 ml) and cooled to 0°C. Chlorosulphonic acid (7.7 ml) was added dropwise to the mixture with continuous stirring, and the temperature was kept below 5°C. After the addition, the mixture was allowed to reach room temperature and 14.5 g of \( p \)-hydroxyacetophenone was added as a suspension in carbon disulphide. The mixture was stirred for 1 h and then left for a further 1 h or overnight. After removal of the lower layer of carbon disulphide and excess of diethylaniline, the brown syrup of the diethylaniline salt of \( p \)-acetylphenyl sulphuric acid was poured slowly with stirring into 30 ml of water containing 15 g of solid KOH. Care was taken to ensure that the temperature did not rise above 35°C and that the final mixture was alkaline. The mixture was extracted with ether (5 \( \times \) 200 ml) to remove the diethylaniline, then it was cooled to 0°C and acidified to pH 4.0 with dilute \( \text{H}_2\text{SO}_4 \). The extraction with ether (5 \( \times \) 200 ml) was then repeated to remove unchanged \( p \)-hydroxyacetophenone. Afterwards, solid \( \text{Ba(OH)}_2 \) was added until the mixture...
became alkaline. The precipitate of BaSO₄ was removed by centrifuging at 3000g for 10 min. Excess of Ba²⁺ was removed from the clear supernatant by passing it through a column (50 cm × 5 cm diam.) of cation-exchange resin (Dowex 50; H⁺ form; 20–50 mesh; Dow Chemical Co., Midland, Mich., U.S.A.) and the acid eluate was collected in a beaker surrounded by ice. The solution was adjusted to pH 10 with aq. 5% (w/v) KOH, then it was concentrated in vacuo at 40°C until potassium p-acetylatedphen sulfatate crystallized out. The crystals were collected by filtration and dissolved in the minimum volume of hot (60°C) water. The ester was then crystallized by the addition of 400 ml of cold ethanol with stirring. After being left at 0°C for 1 h the ester was collected by filtration and was then recrystallized repeatedly from the smallest possible quantity of water until completely free from Cl⁻ and SO₄²⁻ ions. The product was finally dried in vacuo over P₂O₅ at room temperature.

Potassium p-nitrophenyl sulphate. This was prepared by the method employed for potassium p-acetylatedphen sulfatate but substituting an equivalent amount of p-nitrophenol for p-hydroxyacetophenone.

Dipotassium 2-hydroxy-5-nitrophenyl sulphate (4-nitrocatechol sulphate). The crude ester was prepared by the method of Roy (1953) and purified by the method of Dodgson & Spencer (1956).

Animals

Young male Medical Research Council hooded rats (200–250 g body wt.) were used in all experiments.

Methods

Arylsulphatase C preparations. Optimum conditions for arylsulphatase C assay were examined on three liver enzyme preparations. (1) Whole homogenates. Rats were killed by a blow on the head and the livers quickly excised and chilled in ice. After passage through a fine wire mesh to remove connective tissue, the liver pulp was weighed and homogenized for 2 min in ice-cold water in a Tri-R homogenizer (Tri-R Instrument Co. Ltd., Rockville Center, N.Y., U.S.A.; clearance between tube and Teflon pestle, 0.19 mm) rotating at 4000 rev./min, the suspension being forced past the pestle six times. Homogenates (2% wet wt./vol.) thus prepared were suitably diluted with ice-cold water and used for enzyme assay immediately. (2) Fresh microsomal fraction. The liver microsomal fraction was prepared as described by de Duve et al. (1955) except that the mitochondrial and lysosomal fractions were isolated together. The microsomal pellet was suspended in water so that 20 ml of suspension was equivalent to 1 g of liver and was used immediately. (3) Acetone-dried washed microsomal fraction. This was prepared by the method of Dodgson et al. (1959) from the microsomal fraction isolated as described above. The acetone-dried powder was stored at −30°C in a tightly stoppered bottle, under which conditions it retained its enzyme activity for 6 months.

Arylsulphatase A and B preparation. Samples of purified rat liver lysosomal arylsulphatases A and B were provided by Dr. M. Worwood of this department. The enzymes, which had been purified 318 times (A) and 373 times (B) over the original homogenate, were mixed together (12:1, B:A) to obtain a mixture of the enzymes roughly corresponding in composition to that which is extractable from rat liver lysosomes (G. E. R. Hook, unpublished work).

Assay procedures. Arylsulphatase C. The experimental procedure employed for the assay of arylsulphatase C was based on the spectrophotometric method developed by Dodgson & Spencer (1953). In the experiments described below various conditions of assay were explored, but in all cases the basic procedures were similar, particular care being taken to ensure that the extinctions observed were attributable only to the product of the reaction and not to protein or turbidity. Deproteinization with ethanol before absorption measurement, as used by Dodgson et al. (1953), was shown to be applicable to all enzyme preparations irrespective of their protein concentrations. This method is referred to as Method A. With enzyme sources of low protein concentration (microsomal fraction) it was possible to shorten the assay time by omitting the deproteinization with ethanol. In this method the reaction was stopped and the phenolic anion simultaneously developed by the addition of 1M-KOH. Further, precipitation of phosphate, which tended to occur when ethanol was added to reaction mixtures containing phosphate, did not occur. This method (Method B) was used only for the assay of fresh and acetone-dried washed microsomal fraction.

In Method A, enzyme suspension (0.6 ml) adjusted to the appropriate pH was incubated at 37°C for 2 min in a 15-ml tapered centrifuge tube. Substrate solution (80 mm-potassium p-acetylatedphenyl sulphate in 0.2M-Na₂HPO₄-NaH₂PO₄ buffer, pH 8.0, 0.6 ml) was then added and after being mixed, the whole was incubated for 30 min at 37°C. The reaction was terminated and protein precipitated by the addition of 4.8 ml of ethanol. The suspension was mixed thoroughly and left at 0°C for 30 min, then the precipitated material was separated by centrifuging at 2000g for 15 min; 5 ml of the clear supernatant was withdrawn and to this was added 1 ml of 1M-KOH. Determinations were done in duplicate and appropriate controls were employed in which enzyme and substrate were incubated separately and only mixed immediately before addition of ethanol. The extinction of the test solution was then determined at the wavelength of maximum absorption of p-hydroxy-
acetoephene (327 nm, ε = 21,700 litre·mol⁻¹·cm⁻¹ in ethanolic KOH). At the high substrate concentration employed, the absorption caused by the unhydrolysed ester at this wavelength is appreciable and instrument readings are somewhat inaccurate if test and control determinations are made against a water blank as described by Dodgson & Spencer (1953). Extinctions were therefore measured against the appropriate control solution or a 6 mM solution of potassium p-acetylphenyl sulphate in ethanolic KOH. For all practical purposes the contributions to the extinction from small differences in the amounts of unhydrolysed ester present in the tests and controls could be ignored.

Method B was identical with Method A except that after incubation the reaction was stopped and the phenolic anion was developed by the direct addition of 4.8 ml of 1 M KOH. Extinction was then measured at the wavelength of maximum absorption of p-hydroxyacetophenone in aq. 1 M KOH (323 nm, ε = 20,500 litre·mol⁻¹·cm⁻¹) as described above. Although the wavelength of maximum absorption and extinction are slightly different in aqueous and ethanolic KOH, this did not affect the method.

Enzyme activity was calculated as described by Dodgson & Spencer (1953), one unit of activity being defined as the number of nmol of ester hydrolysed/h under these assay conditions. The method also worked satisfactorily on a semi-micro scale with one-fifth of the quantities stated above.

Under these assay conditions, activity was linear with time up to 30 min and with enzyme concentration, and p-hydroxyacetophenone was completely recoverable from the incubation mixtures.

When determinations were made with potassium p-nitrophenyl sulphate, Methods A and B were employed, with 24 mM-potassium p-nitrophenyl sulphate in 0.2 M-Na₂HPO₄–NaH₂PO₄ or 0.2 M-tris-acetic acid buffer, pH 8.0. Extinctions were conveniently measured against a water blank in this case.

Arylsulphatases A and B. The combined activities of arylsulphatases A and B were assayed as follows. Enzyme (0.2 ml) in 0.5 M-sodium acetate-acetic acid buffer, pH 5.5, was incubated with 0.2 ml of 0.02 M-dipotassium 2-hydroxy-5-nitrophenyl sulphate (in the same buffer) at 37°C for 15 min. The reaction was stopped by the addition of 1 ml of 1 M NaOH and the extinction of the liberated 4-nitroacetanilide was measured within 1 h at 515 nm (ε₅₁₅ = 11,200 litre·mol⁻¹·cm⁻¹). Appropriate control determinations were made as usual. One unit of activity is defined as the number of nmol of ester hydrolysed/h under the assay conditions used.

Experimental and Results

Optimum conditions for arylsulphatase C acting on potassium p-acetylphenyl sulphate

pH-activity relationship. Determinations were made on the three preparations of arylsulphatase C by using a final concentration of 40 mM-potassium

---

**Fig. 1. Effect of pH on the arylsulphatase C activity of rat liver preparations**

The activity of whole homogenate (○), fresh microsomal fraction (■) and acetone-dried microsomal fraction (▲) was measured towards potassium p-acetylphenyl sulphate in (a) 0.1 M-tris-acetic acid and (b) 0.1 M-Na₂HPO₄–NaH₂PO₄ buffers. The assay was performed by the appropriate method (Method A or Method B) as described in the text with 80 mM substrate in 0.2 M buffers adjusted to the indicated values.

Vol. 128
The activity of whole homogenates (○), fresh microsomal fraction (■) and acetone-dried microsomal fraction (▲) of rat liver was measured towards potassium p-acetylphenyl sulphate in (a) 0.1M-tris-acetic acid and (b) 0.1M-Na₂HPO₄-NaH₂PO₄ buffers. The assay was performed by the appropriate method (Method A or Method B) as described in the text with substrate of twice the final concentration in 0.2M buffers adjusted to pH 8.0.

In the present experiments (40mM) this would be greater. It was therefore necessary to establish assay conditions in which there could be no contribution from these enzymes. An artificial mixture of arylsulphatases A and B, roughly corresponding to that found in rat liver lysosomes, was used as a test system in these experiments. The enzyme activity of the mixture was first assayed by using dipotassium 2-hydroxy-5-nitrophenyl sulphate in acetate buffer (the normal assay conditions) and was then assayed over the pH range 5.0-9.0 with both potassium p-acetylphenyl sulphate (40mM final concentration) and dipotassium 2-hydroxy-5-nitrophenyl sulphate (20mM), both in 0.1M-Na₂HPO₄-NaH₂PO₄ buffer. Arylsulphatase C cannot be completely separated from arylsulphatases A and B, which contaminate the microsomal fraction, and even the best preparation available (an acetone-dried, washed microsomal fraction) retained appreciable activity (Dodgson et al., 1957). Therefore an aqueous suspension of fresh microsomal fraction was used as the source of arylsulphatase C and was also assayed with potassium p-acetylphenyl sulphate and dipotassium 2-hydroxy-5-nitrophenyl sulphate in phosphate buffer as described above. Finally, the mixture of arylsulphatases A and B containing a known total activity was then added to the preparation of arylsulphatase C and the complete mixture was assayed with potassium p-acetylphenyl sulphate (40mM) and dipotassium 2-hydroxy-5-nitrophenyl sulphate (20mM) over the pH range 5.0-9.0 in 0.1M-phosphate buffer.
buffer. The mixture of arylsulphatases A and B, which had an activity of 4000 units/ml when measured with dipotassium 2-hydroxy-5-nitrophenyl sulphate at pH 5.5 in acetate buffer, had a negligible activity (less than 20%) towards dipotassium 2-hydroxy-5-nitrophenyl sulphate and no activity towards potassium p-acetylphenyl sulphate in 0.1 M-phosphate buffer over the pH range tested. Fig. 3 shows that addition of this mixture to the arylsulphatase C preparation had no effect on the measured activity of the latter towards potassium p-acetylphenyl sulphate in 0.1 M-phosphate buffer. Thus, under the conditions of assay of arylsulphatase C, there was no detectable interference from the presence of considerable quantities of arylsulphatases A and B such as would be encountered in rat liver preparations.

![Fig. 3. Effect of pH on the activity of rat liver fresh microsomal fraction and purified arylsulphatases A and B](image)

**Table 1. Effect of sodium chloride on the arylsulphatase C activity of rat liver fresh microsomal fraction**

Assays were performed by Method B with potassium p-acetylphenyl sulphate or potassium p-nitrophenyl sulphate in 0.1 M-tris-acetic acid or 0.1 M-Na₂HPO₄—NaH₂PO₄ buffers as described in the text.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Final concentration of NaCl (m)</th>
<th>Effect of buffer used (%) of activity in absence of chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.1 M-Tris—acetic acid 0.1 M-Phosphate</td>
</tr>
<tr>
<td>Potassium p-acetylphenyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>0.10</td>
<td>94</td>
<td>99</td>
</tr>
<tr>
<td>0.20</td>
<td>96</td>
<td>99</td>
</tr>
<tr>
<td>Potassium p-nitrophenyl</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>sulphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>123</td>
<td>104</td>
</tr>
<tr>
<td>0.10</td>
<td>127</td>
<td>111</td>
</tr>
<tr>
<td>0.20</td>
<td>129</td>
<td>114</td>
</tr>
</tbody>
</table>
Effect of chloride on the activity of the microsomal fraction towards potassium p-acetylphenyl sulphate and potassium p-nitrophenyl sulphate. Potassium p-nitrophenyl sulphate has been used by various workers for the assay of arylsulphatase C since, although it is also hydrolysed by arylsulphatases A and B which are always found in association with the enzyme, the extent of this is minimal under the assay conditions used. However, chloride, which is often found as a constituent of buffering or fractionation media, has a pronounced activatory effect on the activity of arylsulphatase B towards this substrate, thus rendering the assay of arylsulphatase C with potassium p-nitrophenyl sulphate inaccurate. In developing the modified assay method with potassium p-acetylphenyl sulphate it was therefore necessary to establish that similar problems did not arise. Aqueous suspensions of a fresh microsomal fraction of rat liver were made up to contain various concentrations of NaCl by diluting them with equal volumes of NaCl solutions of twice the final concentration required. The preparations were then assayed for activity towards potassium p-acetylphenyl sulphate (40 mM) and potassium p-nitrophenyl sulphate (24 mM) in 0.1 M-tris-acetic acid and 0.1 M-phosphate buffers. Table 1 shows that the variation in arylsulphatase C activity in the presence of various concentrations of NaCl is negligible only when the determination is made with potassium p-acetylphenyl sulphate in the presence of 0.1 M-phosphate buffer.

Discussion

Much of the reported work on the arylsulphatase enzymes is of only limited value, since due attention has not always been paid to the problems associated with their assay. In the case of arylsulphatase C, the injudicious choice of assay substrate is the main source of error, since p-nitrophenyl sulphate, which is known to be unsuitable in several respects, has been generally employed. The enzyme can, however, be assayed accurately in any rat tissue preparation with p-acetylphenyl sulphate, the substrate originally selected for its assay by Dodgson & Spencer (1953), provided that the reaction is performed in the presence of phosphate.

In addition to its general usefulness in studies on the arylsulphatases, the specific assay method enables full use to be made of arylsulphatase C as a marker enzyme for the rat liver microsomal fraction.

This work was supported by U.S. Public Health Service Research Grant no. AMO 1982-08 from the Arthritis and Metabolic Diseases Division. D. W. M. is grateful to the Medical Research Council for a studentship.

References