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

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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 40mM substrate at pH8.0 in the presence of 0.1M-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 aryl-

sulphatase 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 was 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 × 200 ml) to remove the diethylaniline, then it was cooled to 0°C and acidified to pH 4.0 with dilute H₂SO₄. The extraction with ether (5 × 200 ml) was then repeated to remove unchanged *p*-hydroxyacetophenone. Afterwards, solid Ba(OH)₂ was added until the mixture

became alkaline. The precipitate of BaSO_4 was removed by centrifuging at 3000g for 10 min. Excess of Ba^{2+} was removed from the clear supernatant by passing it through a column (50 cm $\times$ 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*-acetylphenyl sulphate 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 SO_4^{2-} ions. The product was finally dried *in vacuo* over P_2O_5 at room temperature.

Potassium *p*-nitrophenyl sulphate. This was prepared by the method employed for potassium *p*-acetylphenyl sulphate 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 1 M-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*-acetylphenyl sulphate in 0.2 M- Na_2HPO_4 – NaH_2PO_4 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 1 M-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-

acetophenone (327 nm, $\epsilon = 21\,700 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ 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, $\epsilon = 20\,500 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) 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- Na_2HPO_4 - NaH_2PO_4 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-nitrocatechol was measured within 1 h at 515 nm ($\epsilon_{515} = 11\,200 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). 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- Na_2HPO_4 - NaH_2PO_4 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.

Fig. 2. Effect of substrate concentration on the arylsulphatase C activity of rat liver preparations

The activity of whole homogenates ($\circ$), fresh microsomal fraction ($\blacksquare$) and acetone-dried microsomal fraction ($\blacktriangle$) of rat liver was measured towards potassium *p*-acetylphenyl sulphate in (a) 0.1 M-tris-acetic acid and (b) 0.1 M- Na_2HPO_4 - NaH_2PO_4 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 0.2 M buffers adjusted to pH 8.0.

p-acetylphenyl sulphate in 0.1 M-tris-acetic acid or 0.1 M- Na_2HPO_4 - NaH_2PO_4 buffers. Some slight differences in the pH optima for the three enzyme preparations were observed (Fig. 1) although all showed maximum activity at a higher pH value than was observed by Dodgson *et al.* (1953) for whole homogenates (pH 7.2) acting in the presence of sodium acetate-acetic acid buffer.

Substrate concentration-activity relationship. Only slight differences between the affinity of the three enzyme preparations was observed (Fig. 2) under all the conditions used. However, there were noticeable differences between the effects of the two buffer systems used. The K_m with the fresh microsomal fraction was considerably higher in the presence of phosphate buffer (9.0 mM) than in tris buffer (2.5 mM), as were the optimum substrate concentrations (40 and 12 mM respectively). These optima are considerably different from those reported by Dodgson *et al.* (1953) for whole homogenates in sodium acetate-acetic acid buffer (K_m 1.5 mM; optimum substrate concn. 7 mM). The V_{max} in all buffer systems is the same, indicating slight but negligible competitive inhibition of the enzyme by phosphate.

Inhibition of arylsulphatase A and B activity. Although from previous work (Dodgson *et al.*, 1955) it appeared that arylsulphatases A and B probably had only slight activity towards potassium *p*-acetylphenyl sulphate in acetate buffer at low potassium *p*-acetylphenyl sulphate concentrations (7 mM), it seemed likely that at the higher concentration used

in the present experiments (40 mM) 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 (40 mM final concentration) and dipotassium 2-hydroxy-5-nitrophenyl sulphate (20 mM), both in 0.1 M- Na_2HPO_4 - NaH_2PO_4 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 (40 mM) and dipotassium 2-hydroxy-5-nitrophenyl sulphate (20 mM) over the pH range 5.0-9.0 in 0.1 M-phosphate

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

Fresh microsomal fraction was assayed alone (a, b) and in the presence of a mixture of purified rat liver arylsulphatases A and B (c, d) with potassium *p*-acetylphenyl sulphate (a, c) and dipotassium 2-hydroxy-5-nitrophenyl sulphate (b, d) in 0.1 M- Na_2HPO_4 - NaH_2PO_4 buffer. The assay was performed by Method B and the method for arylsulphatases A and B with substrates of twice the final concentrations in 0.2 M- Na_2HPO_4 - NaH_2PO_4 buffer as described in the text.

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- Na_2HPO_4 - NaH_2PO_4 buffers as described in the text.

Substrate	Final concentration of NaCl (M)	Effect of buffer used (% of activity in absence of chloride)	
		0.1 M-Tris-acetic acid	0.1 M-Phosphate
Potassium <i>p</i> -acetylphenyl sulphate	0	100	100
	0.05	90	98
	0.10	94	99
	0.20	96	99
Potassium <i>p</i> -nitrophenyl sulphate	0	100	100
	0.05	123	104
	0.10	127	111
	0.20	129	114

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 (40mM) and potassium p-nitrophenyl sulphate (24mM) 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.

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