Problems Associated with the Assay of Arylsulphatases A and B of Rat Tissues

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A method for the separation and purification of rat liver arylsulphatases A and B by gel filtration on Sephadex G-200 is described. The properties of the A enzyme and its molecular weight are similar to those of the corresponding ox liver enzyme. The B enzymes were found to be dissimilar. The method already developed for the assay of the corresponding enzymes from human tissues was shown to be unsuitable for the assay of the enzymes of rat tissues. A method of assay was developed which permits an approximate determination of the individual rat liver enzymes in a mixture of the two, but precise determination requires prior separation of the enzymes by gel-filtration chromatography.

Early studies on mammalian arylsulphatase enzymes (arylsulphate sulphohydrolase, EC 3.1.6.1) were frequently contradictory and confusing [see, for example, Huggins & Smith (1947) and Maengwyn-Davies & Friedenwald (1954); Dodgson *et al.* (1953) and Roy (1954a)] and the reasons for the various conflicting findings were not apparent until Dodgson *et al.* (1954, 1955, 1956) demonstrated that mammalian livers possessed at least three distinct enzymes capable of hydrolysing arylsulphate esters. Two of these (arylsulphatases A and B; see Roy, 1953a) were associated with a 'mitochondrial' fraction [later shown to be in the lysosomal component of this fraction (Viala & Gianetto, 1955)] and showed high activity towards the chromogenic substrate dipotassium 2-hydroxy-5-nitrophenyl sulphate. The other enzyme (arylsulphatase C; see Dodgson *et al.*, 1955) was insoluble, associated with the 'microsomal' fraction and showed high activity towards potassium *p*-acetylphenyl sulphate.

The difficulties inherent in the independent assay of these enzymes in mammalian tissues were discussed by Dodgson & Spencer (1957) (see also Roy, 1960a) and have recently been re-stated and elaborated by Dodgson & Rose (1970) and by Roy & Trudinger (1970). Briefly, the assay of arylsulphatase C now presents no difficulties, at least as far as rat tissues are concerned (Milsom *et al.*, 1968, 1972; Milsom & Rose, 1970), but the independent assay of

the A and B enzymes is a more formidable problem which has been resolved only for human sources of the enzymes (Baum *et al.*, 1959; see also Austin *et al.*, 1964) and possibly also for those of ox brain (Dzialoszynski *et al.*, 1966).

Reports from our laboratories and that of Dr. A. B. Roy have drawn attention to the assay difficulties, many of which are due to the unusual kinetic behaviour of the enzymes. In spite of this, there is a considerable amount of confusion in the literature and in at least one instance (Whitfield, 1969) the theoretical background to a reported method for determining A and B enzymes in the urine has been misinterpreted (cf. Baum *et al.*, 1958, 1959; Baum & Dodgson, 1958) and temperature properties are attributed to enzyme B which are actually appropriate to enzyme A.

In recent years, arylsulphatases A and B have been increasingly employed as marker enzymes for lysosomes in cell fractionation studies on rat tissues (e.g. Bowers *et al.*, 1967; Ragab *et al.*, 1967; Withers *et al.*, 1968) and in histological work (e.g. Goldfischer, 1965; Hopsu-Havu *et al.*, 1967; Abraham, 1967). In these particular cases the combined activity of the two enzymes is measured, a somewhat unsatisfactory compromise for, apart from obvious kinetic reasons, there has been a claim (Roy, 1960b) that the enzymes may be released from lysosomes at different rates. Moreover, it is well established for ox and human sources (Roy, 1953b; Baum *et al.*, 1958) that the A enzyme does not exhibit normal enzyme concentration-activity relationships. In other studies on rat tissues (e.g. Guha & Roels, 1965; Ugazio, 1960) attempts to measure arylsulphatases A and B have been made employing experimental conditions appropriate to the ox enzyme, in spite of the fact that the

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literature on mammalian arylsulphatase enzymes cites a number of examples of marked species differences between enzymes [e.g. Dodgson *et al.* (1956) and Roy (1954b); Dodgson *et al.* (1957) and Roy (1958)].

The present work describes in detail some of the properties of rat liver arylsulphatases A and B and establishes that they cannot be assayed independently by the methods that are applicable to the corresponding human enzymes.

Materials and Methods

Materials

Dipotassium 2-hydroxy-5-nitrophenyl sulphate was prepared by the method of Dodgson & Spencer (1956). Sephadex G-200 and Blue Dextran 2000 were supplied by Pharmacia (G.B.) Ltd., London W.5, U.K. and Whatman cellulose CM-52 ion-exchanger by W. and R. Balston Ltd., Maidstone, Kent, U.K. The Sephadex column was calibrated with standard proteins supplied by Schwarz/Mann, Orangeburg, N.Y., U.S.A. and all other chemicals were of analytical grade.

Methods

Total arylsulphatase assays. During enzyme purification studies, an approximation of the total combined activities of arylsulphatases A and B present at different stages was obtained as a routine as follows. Enzyme solutions were diluted with water. The incubation mixture consisted of 0.2 ml of diluted solution and 0.2 ml of 0.02 M-dipotassium 2-hydroxy-5-nitrophenyl sulphate in 0.5 M-sodium acetate solution, adjusted with acetic acid to pH 5.6. Incubation was for 10 min at 37°C after which the reaction was stopped by the addition of 2.5 ml of 1 M-NaOH and the E_{515} was measured ($\epsilon = 12000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$).

Protein assay. This was carried out by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Preparation of arylsulphatases A and B. Adult male rats of the Medical Research Council hooded strain were starved for 24 h and killed by a blow on the back of the neck. All subsequent operations were carried out at 4°C. The livers were removed and placed in ice-cold 0.25 M-sucrose (all sucrose solutions contained 1 mM-EDTA and were adjusted to pH 7.0 with 1 M-KOH). The livers were passed through a domestic mincer and 180 g of the minced material was suspended in 500 ml of the 0.25 M-sucrose solution. Homogenization (Waring Blender) was for 20 s at maximum speed and the suspension was diluted to 1350 ml.

Lysosomes were then isolated by a simplified version of the method of Ragab *et al.* (1967). Nuclear and mitochondrial pellets were sedimented by centrifuging in a refrigerated centrifuge at 1000 g_{av} , and

3300 g_{av} , respectively for 10 min and a mitochondrial-lysosomal pellet was then prepared by centrifuging the supernatant at 11700 g_{av} , for 35 min in an MSE High Speed 18 centrifuge. The pellet was gently resuspended in 200 ml of 0.3 M-sucrose and 50 ml portions were layered over discontinuous gradients consisting of 70 ml of 0.6 M-sucrose and 60 ml of 0.45 M-sucrose. After centrifuging at 3300 g_{av} , for 10 min the supernatant was decanted and centrifuged at 11700 g_{av} , for 40 min. As much as possible of the pink microsomal layer was carefully removed from the brown lysosomal pellet and this pellet was resuspended in 0.25 M-sucrose (40 ml).

The lysosomal suspension was frozen and thawed ten times for 5 min periods. The suspension was then centrifuged for 30 min at 80000 g_{av} . (Spinco model L centrifuge, no. 40 rotor) and the supernatant decanted. The sediment was resuspended in 0.25 M-sucrose solution and recentrifuged. The combined supernatants were concentrated to approx. 5 ml by vacuum ultrafiltration and dialysed for 4 days against acetate buffer, pH 5.0, $I = 0.1$ (final concns. were 0.05 M-NaCl, 0.07 M-sodium acetate and 0.03 M-acetic acid). The precipitate was removed by centrifuging at 41000 g_{av} , for 30 min and the supernatant was concentrated to approx. 2 ml.

The A and B enzymes were then separated by gel-filtration chromatography (see the Results and Discussion section). The solution was applied to a column (1.5 cm $\times$ 85 cm) of Sephadex G-200, previously equilibrated with the acetate buffer described above, and eluted with the same buffer. Fractions of 2 ml were collected. The A enzyme appeared immediately after the void volume and the B enzyme much later (Fig. 1). The fractions containing the A and B enzymes were collected and each enzyme solution was concentrated to about 2 ml by vacuum ultrafiltration.

Further purification of arylsulphatase A. The concentrated A enzyme solution was dialysed against barbiturate buffer solution, $I = 0.1$, pH = 7.5 (final concn. 0.09 M-NaCl, 0.01 M-sodium diethyl barbiturate, 0.02 M-barbituric acid) for 4 days. The enzyme solution was applied to a Sephadex G-200 column equilibrated with the barbiturate buffer and eluted with the same buffer. Under these circumstances the enzyme assumes its lower-molecular-weight form (see the Results and Discussion section) thus allowing it to be separated from other material that emerges in the void volume. The active fractions were combined, concentrated to 2 ml, dialysed against water for 24 h and stored at 4°C.

Further purification of arylsulphatase B. The solution of the B enzyme was dialysed for 36 h against 0.01 M-sodium acetate solution (adjusted to pH 5.25 with acetic acid) and applied to a column (0.9 cm $\times$ 15 cm) of cellulose CM-52 that had been equilibrated with the same buffer. The column was eluted with

Fig. 1. Gel filtration on Sephadex G-200 of a soluble lysosomal extract

See the Materials and Methods section for details. Arylsulphatase activity: ●, E_{515} ; ▲, E_{280} .

Table 1. Purification of rat liver arylsulphatases A and B

The total activity is expressed as μmol of dipotassium 2-hydroxy-5-nitrophenyl sulphate hydrolysed/10 min under the assay conditions described in the Materials and Methods section. Values are means of three purifications.

Stage	Total activity (units)	Specific activity (units/mg of protein)
Homogenate (from 180 g of liver)	4943	0.134
Lysosomal fraction	1162	1.46
Soluble lysosomal extract	770	4.55
Dialysis, pH 5.0	470	14.8
Sephadex G-200 chromatography at pH 5.0 A enzyme	125	63
B enzyme	112	16.5
Sephadex G-200 chromatography at pH 7.5 of A enzyme	61	90
Cellulose CM-52 chromatography at pH 5.25 of B enzyme	34*	51

* Single value.

buffer and fractions of 3 ml were collected. After eight fractions, elution was continued with a linear gradient formed from the acetate buffer and the same buffer containing 0.3 M-NaCl. The enzyme was eluted when the ionic strength of the buffer was between 0.1 and 0.2 and the active fractions were pooled, concentrated to 2 ml (vacuum ultrafiltration) and dialysed for 24 h against water. The enzyme was stored at 4°C.

Estimation of molecular weights. The method of Andrews (1965) was employed with Sephadex G-200 columns that had been calibrated with Blue Dextran 2000 and standard proteins.

Results and Discussion

Enzyme purification

Flynn (1966) has shown that rat liver arylsulphatases A and B can be separated by gel-filtration chromatography on Sephadex G-200 ($I = 0.1$, pH =

5.0). Two peaks containing arylsulphatase activity were obtained: one at the void volume and a slower-moving component. The former peak was identified as arylsulphatase A and the latter as the B enzyme. At pH 7.5 ($I = 0.1$) the A enzyme changed to a lower-molecular-weight form. The purification procedure described in the present paper is based on these properties. The mean values of results obtained from three preparations are given in Table 1. The procedure provided separate preparations of enzymes A and B of sufficient purity for the studies envisaged. Polyacrylamide-gel electrophoresis showed the presence of several bands of inactive protein in addition to the active enzymes.

Molecular weights of the enzymes

These were determined by gel-filtration chromatography. At pH 7.5 the A enzyme had an elution volume corresponding to a molecular weight of

130000, but at pH 5.0 the apparent molecular weight was approx. 400000. Nichol & Roy (1965) have demonstrated that the ox liver A enzyme exists as a monomer of molecular weight 107000 at pH 7.5 ($I = 0.1$) and as a tetramer of molecular weight 411000 at pH 5.0 ($I = 0.1$). The rat and ox liver enzymes therefore appear to be similar as regards molecular weight and ability to polymerize. At both pH 7.5 and 5.0 rat arylsulphatase B showed an asymmetric elution pattern corresponding to a range of molecular weights from 34000 to 66000. The rat liver B enzyme is very different from the highly purified ox liver enzyme (cf. Allen & Roy, 1968). Thus the ox B enzyme had an estimated molecular weight of 25000 and was strongly absorbed by Sephadex G-200 at pH 5.0 and could not be completely eluted.

Kinetic properties of the enzymes

Arylsulphatases A and B from all known mammalian sources exhibit anomalous kinetic behaviour towards dipotassium 2-hydroxy-5-nitrophenyl sulphate. The anomalies are particularly striking in the case of the former enzyme (see Dodgson & Rose, 1970). Table 2 summarizes the behaviour of the two rat liver enzymes under a variety of experimental conditions, some of which will be referred to at greater length in subsequent sections. The rat liver A enzyme shares many properties in common with the ox liver enzyme (see Nichol & Roy, 1965). The optimum pH is 5.6 under initial reaction conditions for the two enzymes and the optimum substrate concentrations are similar. These similarities also persist when longer incubation times are employed (60 min) although the apparent pH optimum is then shifted owing to inactivation at pH 5.6. The situation with the B enzymes is rather different in that the rat and human enzymes share a common pH optimum, 5.9–6.0 (see Dodgson

& Wynn, 1958), which is higher than that of ox enzyme (pH 5.4, see Allen & Roy, 1968).

Under suitable conditions (Fig. 2a) rat arylsulphatase A shows the anomalous relationship between time and enzyme activity found with the corresponding human enzyme (Baum *et al.*, 1958) but the effect is not nearly so marked. With both enzymes these anomalies are maximal at the respective optimum conditions for the initial reaction. An explanation of the anomalies exhibited by human A enzyme has been offered by Baum & Dodgson (1958) and this has recently been confirmed for the corresponding ox enzyme (Nicholls & Roy, 1971). These particular anomalies are not a feature of arylsulphatase B, which exhibits a normal relationship between incubation time and activity for periods of up to 2 h.

Baum *et al.* (1958) have previously described the effects of temperature on the anomalous time-course of the reaction of human liver arylsulphatase A. When long incubation periods (1 h) were employed the enzyme apparently exhibited higher activity at 20.5°C than at 37.5°C. Analogous effects of temperature are exhibited by the rat enzyme (Fig. 3) when incubated over the same period. Arylsulphatase B does not show this unusual behaviour. It is noteworthy that the values for the pH optimum and K_m for the A enzyme at 15°C are the same as those obtained under initial reaction conditions (i.e. 5 min incubation period) at 37°C (Table 2). The anomalous kinetic behaviour of the enzyme is not observed at 15°C (Fig. 2b).

Conditions for the assay of the A and B enzymes from rat liver

The only valid and well-tested method for assaying A and B enzymes independently has been that of Baum *et al.* (1959), which was developed for measuring

Table 2. *Kinetic properties of rat liver arylsulphatases A and B*

For details see the text.

Enzyme	Incubation period (min)	Incubation temp. (°C)	Optimum pH	K_m (mM)
A	5	37	5.6	0.60
	60	37	4.9	*
	60	15	5.6	0.60
B	5	37	5.9	1.81
	60	37	5.9	1.04
	60	15	5.9	†

* Activity at 37°C and pH 4.9 was not linear with time; maximum activity was at 2 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate.

† Pronounced inhibition by excess of substrate; maximum activity was at 3 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate.

Fig. 2. Time-activity curves for different concentrations of rat liver arylsulphatase A acting on 5 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate in the presence of 0.25 M-sodium acetate-acetic acid buffer, pH 5.6, at (a) 37°C and (b) 15°C

For details see the text. The enzyme dilutions were as follows: ●, $\times 18$; □, $\times 36$; ○, $90\times$; ■, $\times 240$.

the enzyme activities in human urine. In this method samples are first dialysed and the A enzyme is then assayed at pH 5.0, at low substrate concentration (5 mM-dipotassium 2-hydroxy-5-nitrophenylsulphate) and in the presence of 0.25 mM- $\text{Na}_4\text{P}_2\text{O}_7$ and 0.86 M-NaCl (A reagent). Under these conditions normal kinetics are exhibited by the human A enzyme and B enzyme activity is 2% of that given under conditions described below for the assay of the B enzyme. The B enzyme is assayed at pH 6.0 in the presence of 0.025 M-dipotassium 2-hydroxy-5-nitrophenyl sulphate and 0.055 M-barium acetate (B reagent). Under these conditions the activity of the A enzyme virtually ceases after the first 30 min of incubation (see Baum & Dodgson, 1958) and B enzyme activity can then be calculated from the amount of substrate hydrolysed between 30 and 90 min. The complete method has also been successfully applied to human tissues (e.g. Austin *et al.*, 1964; Mehl & Jatzkewitz, 1965) and its application led directly to the discovery of the relationship between meta-chromatic leucodystrophy and arylsulphatase A.

In spite of the differences exhibited by the two rat enzymes (see Table 2) it is not practicable to measure the activity of either enzyme in the presence of the other merely by choosing a different pH and substrate concentration. Thus the pH-activity curves overlap each other to a considerable extent; moreover, the A enzyme, over incubation periods of more than a few minutes, shows an anomalous relationship between enzyme concentration and activity (see Fig. 2a). It was therefore necessary to establish whether the method devised for the human enzymes by Baum *et al.* (1959) could be applied to rat tissues.

In the first instance the effects on the rat enzymes of various components present in the assay reagents were investigated. The behaviour of the A enzyme towards these components was virtually identical with that of human A enzyme. Thus 0.1 mM- $\text{Na}_4\text{P}_2\text{O}_7$ abolished the anomalous time-activity curve whereas NaCl did not inhibit the enzyme at concentrations up to 0.86 M. Unfortunately, rat arylsulphatase B, in contrast with human arylsulphatase B, still exhibited appreciable activity when measured in the presence

Fig. 3. Effect of temperature on arylsulphatases A and B

The enzyme solutions (0.2ml) were incubated for 1 h with 0.2ml of 20mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate in 0.5M-sodium acetate buffer adjusted to pH 5.6 (A enzyme) and 6.0 (B enzyme) with acetic acid. ○, Arylsulphatase A; □, arylsulphatase B.

of these compounds (i.e. with the reagent for the A enzyme). Thus the activity of the B enzyme was still about 30% of that obtained at its optimum conditions in the absence of any inhibitor. The explanation for this seems to reside in the failure of $\text{Na}_4\text{P}_2\text{O}_7$ to inhibit the rat B enzyme to an extent comparable with that obtained with the human arylsulphatase B. The method for the assay of human arylsulphatase A in the presence of the B enzyme is therefore not applicable to the rat enzyme.

It seemed possible that the A enzyme activity of the rat might be measured independently of B activity by taking advantage of the temperature effect illustrated in Fig. 3. Thus the A enzyme, incubated for 1 h under the conditions appropriate for the initial reaction, showed slightly greater activity at 15°C than at 37°C. In contrast the B enzyme exhibited comparatively little activity at the lower temperature. Unfortunately, the activity of A enzyme at 15°C was not linear over a 60min incubation period (Fig. 2b) and it was necessary to modify experimental conditions to resolve this problem. Incubation at 15°C and pH 5.3 in the presence of a buffer of low ionic

strength (0.01M-dipotassium 2-hydroxy-5-nitrophenyl sulphate in 0.025M-sodium acetate and 0.025M-NaCl) gave linear time-activity and enzyme-activity curves. The A enzyme activity over a 60min period was then approximately equal to that exhibited at 37°C under the optimum conditions appropriate for the initial reaction at this temperature (0.01M-substrate, 0.25M-sodium acetate buffer adjusted to pH 5.6 with acetic acid). In the assay procedure 0.2ml portions of enzyme were incubated at 15°C for 1 h with an equal volume of the buffered substrate solution and the reaction was stopped and colour developed by the addition of 2.5ml of 1.0M-NaOH. Extinction was measured at 515nm. Appropriate controls were made in which enzyme and substrate were incubated separately and only mixed immediately before the addition of alkali. Rat arylsulphatase B activity measured under the conditions for A enzyme was about 5% of the activity that it would normally have given under its own optimum conditions. When a mixture of equal amounts of each enzyme (i.e. equal quantities of substrate hydrolysed/h with A enzyme being measured at pH 5.3 and B enzyme at pH 6.0, both in the presence of 0.01M-dipotassium 2-hydroxy-5-nitrophenyl sulphate in 0.25M-sodium acetate-acetic acid buffer) was assayed for arylsulphatase A activity at 15°C, the recoveries of A enzyme were virtually identical ($\pm 5\%$) with the values obtained when only the A enzyme was present.

For the assay of rat arylsulphatase B the directions of Baum *et al.* (1959) were found to be suitable, provided that barium acetate was omitted from reagent B. The function of barium acetate in the original assay procedure was to remove SO_4^{2-} ions from the incubation mixture with the consequent virtual cessation of the human arylsulphatase A activity at stage II of its time-course (see Baum & Dodgson, 1958). Unfortunately, unlike the human B enzyme, rat arylsulphatase B was significantly inhibited by barium acetate after 60min of incubation. However, the presence of the latter in the incubation mixture was not necessary, as stage II of the time-course of the activity of rat A enzyme persisted, provided that the concentration of the enzyme was kept sufficiently low (see below). The assay procedure adopted was as follows. Enzyme solution (0.5ml) was incubated at 37°C with an equal volume of 0.02M-dipotassium 2-hydroxy-5-nitrophenyl sulphate in 0.5M-sodium acetate solution adjusted to pH 6.0 with acetic acid. After 30min and 90min, 0.4ml portions were withdrawn, added to 2.5ml of 1.0M-NaOH and the extinction of the solution measured as usual. Appropriate control determinations were made. The corrected spectrophotometric readings ($E_{30\text{min}}$ and $E_{90\text{min}}$) were plotted against time and a line through these points was extrapolated back to zero time. The intercept so obtained is an approximate measure of any arylsulphatase A

Table 3. Measurement of rat liver arylsulphatase A activity in mixtures of the purified A and B enzymes

Enzyme activities are μmol of dipotassium 2-hydroxy-5-nitrophenyl sulphate hydrolysed/h under the following conditions: A enzyme activity was measured at 15°C as described in the Results and Discussion section; B enzyme activity was measured under optimum conditions at 37°C (Table 2).

Ratio A enzyme/B enzyme activities in the mixture	Added A enzyme activity	Measured A enzyme activity	% Difference
3.3	0.160	0.165	3.2
1.7	0.165	0.164	0.5
1.2	0.111	0.114	3.0
0.6	0.109	0.118	8.2
0.2	0.059	0.073	24
0.1	0.010	0.013	30

present. If the enzyme solutions employed are so diluted that the value of this intercept is less than 0.1 the A enzyme makes no contribution to enzyme activity between 30 and 90 min and the amount of B enzyme can then be calculated from the difference between $E_{90\text{ min}}$ and $E_{30\text{ min}}$ (cf. Baum *et al.*, 1959). Employing this procedure the difference between the added B enzyme activity and the estimated activity of B enzyme in mixtures of the two enzymes (when the ratio of A enzyme/B enzyme activities ranged from 0.2 to 2.2) was 4.0 ± 2.7 (s.d.) %.

Although, at first sight, it now seemed that a suitable method for the independent assay of rat A and B enzymes had been devised, further studies revealed that the method could not be applied to rat tissues or tissue extracts. The reason for this is that rat tissues, in contrast with those of humans contain a large relative excess of the B enzyme (Hook *et al.*, 1973). The effect of this is that B enzyme can be measured without difficulty, but its contribution to the apparent activity of A enzyme in the assay procedure for the latter now becomes significant. This is demonstrated in Table 3, which records the results of experiments designed to check the recoveries of the A enzyme from artificial mixtures. Although good recoveries of A enzyme were obtained at A enzyme/B enzyme ratios greater than 1, at values lower than this the activity contributed by the B enzyme is significant. For this reason it was found necessary to partially separate the enzymes before using the assay procedures described in the present paper and the use of such a method for estimating the activity of the A and B enzymes in tissue extracts is described in the following paper (Hook *et al.*, 1973).

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