Relative Distribution of Arylsulphatases A and B in Rat Liver Parenchymal and Other Cells

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The relative activities of arylsulphatases A and B were measured in rat liver parenchymal and non-parenchymal cells, in peritoneal macrophages and in a number of rat tissues. Although absolute values cannot be obtained, it was shown that the arylsulphatase B/arylsulphatase A activity ratio is much higher in non-parenchymal cells than in parenchymal cells. The ratios in adrenals, brain and testis are very similar to each other but differ from those found in spleen, kidney and liver. These ratio variations may be caused by alterations in the activity of the B enzyme rather than the A enzyme. The relatively high B enzyme/A enzyme ratios in all rat tissues explains why the method devised for the independent assay of human arylsulphatases A and B cannot be employed with rat tissues.

The present concept of lysosomal enzymes assigns them to the role of degrading macromolecules already present in the cell or entering the cell by endocytosis. In this scheme of things lysosomal arylsulphatase A is concerned in the degradation of cerebroside sulphate esters but no function has yet been ascribed to the corresponding B enzyme. Work in these laboratories has so far failed to implicate the enzyme in the degradation of mucopolysaccharides, in the desulphation of the L-tyrosine *O*-sulphate residues of fibrinogen or in the degradation of adenylyl sulphate or 3'-phosphoadenylyl sulphate. There are some indications from studies on human, ox and rat tissues that the two enzymes are quite independent of each other. Thus the disappearance of arylsulphatase A from the tissues of human patients with metachromatic leucodystrophy is not accompanied by any significant variation in the amounts of the B enzyme (Austin *et al.*, 1964). Similarly, there is some indication that the enzymes may be released from lysosomes at different rates (Roy, 1960). These and other observations raise the possibility that the two enzymes might not necessarily have the same significance in different types of cells. Indeed it has never been established that all cell types contain both enzymes.

The present paper describes a method for comparing the ratios of activities of arylsulphatases A and B

in different types of cell and demonstrates that considerable variations in these ratios do occur.

Materials and Methods

Animals

Adult male rats (150–200 g body wt.) of the Medical Research Council hooded strain were used throughout.

Materials

Dipotassium 2-hydroxy-5-nitrophenyl sulphate was prepared by the method of Dodgson & Spencer (1956). Standard proteins for the molecular-weight calibration of Sephadex G-200 were supplied by Schwarz/Mann, Orangeburg, N.Y., U.S.A. Collagenase (*Clostridium histolyticum*) type 1 and hyaluronidase (bovine testes) type 1 were supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A. Nylon mesh (61 μ m and 20 μ m aperture sizes) was obtained from Henry Simon Ltd., Stockport, Chesh., U.K.

Methods

Cell preparations from liver. The method employed for the isolation of parenchymal cells was essentially that of Howard *et al.* (1967) with minor modifications. The liver was removed under ether anaesthesia and perfused with 20 ml of ice-cold calcium-free Hanks solution, followed by 20 ml of mixed enzyme solution (0.05% collagenase and 0.1% hyaluronidase in calcium-free Hanks solution). The liver was finely

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chopped with a razor and then transferred to a 100ml measuring cylinder containing a further 50ml of mixed enzyme solution. The cylinder was then kept at 37°C for 1 h and the contents were gently agitated by bubbling O₂+CO₂ (95:5) through the medium. The suspension was filtered through nylon mesh (61 µm aperture size) and centrifuged at 4°C and 250g for 15 min (MSE 6L centrifuge). The sedimented material was washed twice by re-suspending in 20ml of calcium-free Hanks solution and re-centrifuging as before. The washed material was re-suspended again in 20ml of calcium-free Hanks solution and the parenchymal cells were sedimented by centrifuging at 4°C and 50g for 2 min. The supernatant suspension was retained and the cells were washed three times with the Hanks solution (20ml portions) with intermediate centrifuging and were finally suspended in 10ml of the same solution.

The suspension remaining after removal of the parenchymal cells contained numerous Kupffer cells and other endothelial cells, as well as a large amount of cell debris. The suspension was centrifuged at 50g for 2 min at 4°C to remove any remaining parenchymal cells. The supernatant suspension was removed by pipette and centrifuged at 250g and 4°C for 15 min. The sediment was re-suspended in 25ml of calcium-free Hanks solution and layered on to a discontinuous sucrose gradient which had also been prepared in the Hanks solution. The gradient consisted of three 25ml layers (sp. gravity 1.105, 1.076, 1.050) and the whole was then centrifuged at 250g and 4°C for 15 min. Cells sedimented to the bottom layer and most of the cell debris was retained in the upper layers. The cells were washed three times with calcium-free Hanks solution (20ml portions) with intermediate centrifuging at 250g for 10 min, the cell suspension being filtered through 20 µm nylon mesh before the last centrifugation. The cells were finally suspended in 10ml of the medium.

Peritoneal macrophages. These were obtained from adult male rats that had been injected intraperitoneally 4 days previously with 2ml of 1% glycogen in 0.15M-NaCl. Animals were killed and 20ml of calcium-free Hanks solution was injected into the intraperitoneal cavity. The abdomen was opened and the exudate fluid was removed, filtered through nylon mesh (61 µm) and centrifuged at 4°C and 250g for 15 min. The cells were washed three times with calcium-free Hanks solution with intermediate centrifuging and they were finally suspended in 10ml of the solution.

Cell-counting and viability. Cells were counted with the aid of a haemocytometer and their viability assessed by staining with 0.2% Trypan Blue.

Cell-disruption and preparation of the 'soluble' fraction. Dispersed cells were disrupted by means of the MSE 100W Ultrasonic Disintegrator operating at maximum power for four 1-min periods. Tempera-

ture was maintained below 10°C with the aid of an ice-salt bath. The suspension was then centrifuged at 76000g and 4°C for 1 h on the Spinco L2-65B centrifuge (SW 41 rotor). The supernatant was collected by pipette and dialysed against Tris-HCl buffer ($I=0.02$, pH7.4) for 48 h at 4°C. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Preparation of 'soluble' fraction of organs. Organs were homogenized (MSE homogenizer operating at maximum speed for 5 min) in Tris-HCl buffer ($I=0.02$, pH7.4). The homogenate (10%, w/v) was centrifuged at 76000g for 1 h and the supernatant was dialysed against four changes of Tris-HCl buffer for 48 h (total vol. of 2 litres). All operations were carried out at 4°C.

Total arylsulphatase assay. Total arylsulphatase activity of dialysed 'soluble' fractions was determined and related to the organ or cell. Considerable increase (several-fold) in enzyme activity is observed after dialysis because of the removal of inhibitory ions. Dialysed enzyme solutions were diluted appropriately with water before assaying as described for total arylsulphatase by Worwood *et al.* (1973).

Ratio of arylsulphatase A and B activities. The preceding paper (Worwood *et al.*, 1973) has emphasized the present inability to measure arylsulphatases A and B independently when both are present in rat tissue extracts. It was therefore necessary to separate the two enzymes before applying the assay method appropriate to each enzyme. The following procedure was employed.

The soluble fraction from cells or organs was applied to a column (15 cm × 1 cm) of DE-52 cellulose that had been equilibrated with Tris-HCl buffer, pH7.4, $I=0.02$. The amount of soluble fraction placed on the column was such that it would have hydrolysed between 0.3 and 5 µmol of dipotassium 2-hydroxy-5-nitrophenyl sulphate/min in the total arylsulphatase assay method described above. Both enzymes were initially retained on the column and the B enzyme was then eluted with the same buffer but containing 0.125M-NaCl. Subsequently the A enzyme was eluted by increasing the concentration of NaCl to 0.25M. In practice, after loading the column, 40 fractions of 5ml were collected. The buffer containing 0.125M-NaCl was introduced after fraction 5 had been collected, whereas the buffer containing 0.25M-NaCl was introduced after fraction 19. The eluted B enzyme was always completely free from the A enzyme but the A enzyme was generally contaminated with traces of B enzyme and due allowance was made for this in calculating enzyme activities. In the early stages of the investigation each fraction collected was assayed for A and B enzyme activities and the individual values were summated. However, with practice it became possible to pool the appropriate fractions

containing arylsulphatase A (generally fractions 20–40 and designated 'A pool') and those containing arylsulphatase B (generally fractions 5–19 and designated 'B pool').

Portions (0.25 ml) of each pool were incubated with 0.25 ml of 0.02 M-dipotassium 2-hydroxy-5-nitrophenyl sulphate in 0.5 M-sodium acetate buffer under the following conditions (cf. Worwood *et al.*, 1973): (I) adjusted to pH 6.0 with acetic acid (30 min incubation at 37°C); (II) adjusted to pH 6.0 with acetic acid (90 min incubation at 37°C); (III) adjusted to pH 5.3 with acetic acid and containing 0.05 M-NaCl (60 min incubation at 15°C). In each case the reaction was terminated by addition of 1 M-NaOH and the E_{515} was measured ($\epsilon = 12000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). For the A pool the activities towards the three substrate solutions described above are denoted by the symbols AI, AII and AIII respectively. BI, BII and BIII are the analogous symbols denoting activities given by the B pool.

Considering the B pool first, no A enzyme is present and the arylsulphatase activity (all activities are expressed as μmol of dipotassium 2-hydroxy-5-nitrophenyl sulphate hydrolysed/60 min at 37°C) of this pool is given by the expression: $(\text{BII} - \text{BI})$. The arylsulphatase B activity present in the A pool is given by the expression: $(\text{AII} - \text{AI})$. Thus the total arylsulphatase B activity of both pools is: $(\text{BII} - \text{BI}) + (\text{AII} - \text{AI})$.

With the A pool the situation is complicated because of the presence of the B enzyme. The arylsulphatase A activity of this pool is obtained by subtracting the contribution made by arylsulphatase B from the apparent value obtained for enzyme A with substrate AIII. This arylsulphatase B contribution can be deduced from the expression:

$$\frac{(\text{AII} - \text{AI})\text{BIII}}{(\text{BII} - \text{BI})}$$

Arylsulphatase A activity of the A pool is therefore given by the expression:

$$\text{AIII} - \frac{(\text{AII} - \text{AI})\text{BIII}}{(\text{BII} - \text{BI})}$$

The ratio of B enzyme/A enzyme activity is therefore:

$$\frac{(\text{BII} - \text{BI}) + (\text{AII} - \text{AI})}{\left(\text{AIII} - \frac{(\text{AII} - \text{AI})\text{BIII}}{(\text{BII} - \text{BI})}\right)}$$

It must be emphasized that the method does not give an absolute measure of the two enzymes since the activities are measured under arbitrary conditions. The ratios obtained are not ratios of enzyme molecules and the method is valid only when used for comparative purposes. Recoveries of both enzymes from the cellulose DE-52 column are the same and average $83.0\% \pm 2.4$, S.E.M. (20).

Results and Discussion

Cell preparations

Good preparations of parenchymal cells were obtained; homogeneity varied between 90 and 95% whereas viability was somewhat variable and ranged from 70 to 90%. Measurement of protein after sonication showed that 10^6 cells was equivalent to 2.4 mg of protein. The yield of cells varied somewhat but was of the order of 2.8×10^6 cells/g of rat liver. Total soluble arylsulphatase activity per cell was $5.6 \times 10^{-8} \pm 0.7 \times 10^{-8}$ S.E.M. (7) μmol of dipotassium 2-hydroxy-5-nitrophenyl sulphate hydrolysed/min.

The cells remaining after removal of parenchymal cells were then isolated on a discontinuous sucrose gradient as described above. They were a mixed population including a small number of erythrocytes and attempts made to separate them into further groups by isopycnic centrifugation were not successful. From the literature it is not at all clear as to the precise number of cell types present in liver, although there appear to be at least two main types of sinusoidal cells (Bloom & Fawcett, 1962) as well as parenchymal and biliary cells. In the present study the non-parenchymal cells could be roughly divided into two groups according to cell diameter. Cells with diameters of 5–10 μm were classified as group 1 cells. The average diameter was about 7 μm . The diameters of cells classified as group 2 ranged from 10 to 15 μm . When stained with Haematoxylin and Eosin the group 1 cells appeared to be mainly endothelial cells of the type described by Bloom & Fawcett (1962), as their nuclei stained extremely darkly and details could not be discerned. The larger type of endothelial cells described by those workers, having an oval nucleus and small prominent nucleolus, were also present in appreciable amounts and there were also some polymorphonuclear leucocytes. These cells, when viable, were not distinguishable from each other while mounted in the haemocytometer and hence they were all grouped together under group 1.

Group 2 cells were mainly Kupffer cells as judged by the fact that, in separate experiments, a great many of them contained carbon particles after injection of India ink into the tail vein of the rat before separation of the cells.

The yield of group 1 cells was $2.83 \times 10^5 \pm 1.26 \times 10^5$, S.E.M. (5)/g of rat liver, whereas that of group 2 cells was $2.11 \times 10^5 \pm 0.94 \times 10^5$, S.E.M. (5)/g. The mean total soluble arylsulphatase activity of cells in these preparations was $1.8 \times 10^{-8} \pm 0.6 \times 10^{-8}$, S.E.M. (6) μmol of dipotassium 2-hydroxy-5-nitrophenyl sulphate hydrolysed/min. This mean value does not indicate the activities of the individual cell types but it does indicate that the activities are of the same order of magnitude as the arylsulphatase activity of the parenchymal cell.

Table 1. *Apparent ratios of arylsulphatase B activity to arylsulphatase A activity in rat liver cell preparations and in peritoneal macrophages*

See the text for details.

Cell preparation	Preparation no.	Composition (%)			B enzyme/A enzyme activity ratio
		Group 1	Group 2	Parenchymal	
Parenchymal cells	—	—	—	100	13
Other liver cells	1	48	41	10	25
	2	77	23	—	33
	3	46	49	—	36
	4	86	11	3	49
	5	83	15	—	67
Peritoneal macrophages	—	—	—	—	27

Table 2. *Apparent ratios of arylsulphatase B activity to arylsulphatase A activity in various tissues of adult male rats*

See the text for details.

Tissue	Apparent arylsulphatase activity				B enzyme/A enzyme activity ratio
	(per g of tissue)		(per whole organ)		
	Enzyme B	Enzyme A	Enzyme B	Enzyme A	
Adrenal	272.0	22.1	10.0	0.8	12
Brain	106.0	9.1	148.0	12.7	12
Testis	58.9	5.2	82.3	7.2	11
Spleen	269.0	9.2	184.0	6.3	29
Kidney	410.0	12.4	359.0	10.9	33
Liver	226.0	11.2	2237.0	111.0	20

Ratios of arylsulphatase A and B activities in liver cell preparations

The results given in Table 1, apart from those appertaining to parenchymal and peritoneal cells, represent five separate attempts to isolate the non-parenchymal cells of the liver. As mentioned previously the preparations obtained consisted mainly of two cell-types when cells were classified according to their diameters.

In parenchymal cells the B enzyme/A enzyme activity ratio is 13. However, the corresponding ratio for the non-parenchymal cell preparations is considerably higher. The non-parenchymal cell preparations were sometimes contaminated with erythrocytes to the extent of 1 or 2%. However, this contamination can be ignored for all practical purposes since separate experiments showed that preparations of washed erythrocytes had arylsulphatase activities below 4×10^{-13} μmol of dipotassium 2-hydroxy-5-nitrophenyl sulphate hydrolysed/min per cell. These washed preparations were contaminated with up to 0.5% of cells with a granular appearance and it is possible that the detectable arylsulphatase activity arose entirely from these.

The peritoneal macrophage preparation consisted of 80% of large mononuclear cells (average cell diam., 15 μm) and 15% of small mononuclear cells. About 5% were polymorphonuclear leucocytes. Analysis of the preparation gave a B enzyme/A enzyme activity ratio of 27.

If the B enzyme/A enzyme ratio is considered as a function of cell composition (Table 1) it would appear that the ratio is highest in those preparations that contain the greatest proportion of group 1 cells. It is possible, though it cannot be stated with certainty, that these cells may contain only very small amounts of arylsulphatase A. It is also clear from Table 1 that the B enzyme/A enzyme ratio in the parenchymal cells is considerably lower than the ratios observed in the non-parenchymal cell preparations.

Ratios of arylsulphatases A and B in tissues

The B enzyme/A enzyme ratios in various different rat tissues are recorded in Table 2. The ratios in testis, adrenals and brain are very similar to each other and to that observed in parenchymal cells. Interestingly, the ratio in whole liver (20) is much higher than that

of the parenchymal cell. However, direct comparisons between the results in Tables 1 and 2 must be done with caution because whole cells were disrupted by sonication whereas whole organs were homogenized. Both techniques released virtually all the soluble arylsulphatase from the lysosomes, as further disruption of the pellet remaining after centrifuging the preparations yielded very little (less than 2% of the total) additional soluble material. However, there is no guarantee that preferential inactivation could not occur with one of the techniques.

Of course, a higher B enzyme/A enzyme ratio of whole liver is to be expected because of the contribution of non-parenchymal cells to the soluble arylsulphatase activity. The latter cell-types may contribute as much as 20% of the total soluble arylsulphatase activity of whole liver, an estimate based on the values of Daoust (1958) for the parenchymal cell population (61%) and on the relative total soluble arylsulphatase activities in parenchymal cells and in the remaining cells.

Table 2 also shows the relative amounts of the A and B enzyme activities with respect to the tissue weight and to the organ as a whole. Of course, the liver being the largest organ contains by far the greatest amounts of the A and B enzymes. At the other extreme of the organs tested the adrenals contained the least. Apart from the adrenals and the testis the amount of arylsulphatase A/g of tissue is surprisingly constant. The B enzyme is rather more variable; for example, the kidney has seven times the concentration of B enzyme than has the testis. Perhaps this indicates that the ratio differences noted in this work may be caused by variations in the quantities of the B enzyme rather than fluctuations of A enzyme.

The preponderance of the B enzyme in rat tissues now explains why the method developed by Baum *et al.* (1959) and further investigated by Worwood *et al.* (1973) for the independent assay of human arylsulphatases A and B cannot be applied to rat tissues. For the same reason attempts to differentiate arylsulphatases A and B in, for example, rat nervous tissue (Choy & Cravioto, 1968), by employing histochemical methods based on pH-optima differences are unlikely to be of much value.

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