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Comparative Activity of Arylsulphatases A and B on Two Synthetic Substrates

By EDGARD E. DELVIN,* ANGELA POTTIER and FRANCIS H. GLORIEUX
Genetics Unit, Montreal Shriners Hospital for Crippled Children, Departments of Surgery and Pediatrics, McGill University, Montreal, P.Q. H3G 1A6, Canada

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Rat liver and human skin fibroblasts arylsulphatase A and B activities on both 4-methylumbelliferyl sulphate and 4-nitrocatechol sulphate were compared. The intracellular distribution of activity differed markedly when 4-methylumbelliferyl sulphate was used from that observed with 4-nitrocatechol sulphate. No discrimination between control and metachromatic leucodystrophy or mucopolysaccharidosis (type VI) could be achieved when 4-methylumbelliferyl sulphate was used as substrate. These results contrast sharply with those obtained with 4-nitrocatechol sulphate and cast doubt on the validity of 4-methylumbelliferyl sulphate as substrate for the determination of arylsulphatase A and B activities.

Although the physiological substrates for arylsulphatases A and B (EC 3.1.6.1) have been assigned (Mehl & Jatzkewitz, 1968; Matalon *et al.*, 1974), the activity of these enzymes, which are deficient in metachromatic leucodystrophy (Austin *et al.*, 1965) and mucopolysaccharidosis (type VI) (Beratis *et al.*, 1975; Fluharty *et al.*, 1975) respectively, is usually measured colorimetrically with 4-nitrocatechol sulphate as substrate (Baum *et al.*, 1959). Because of the poor sensitivity of the method, inaccurate results are obtained at low concentration of enzyme. Hence for diagnostic purposes, a more sensitive assay is required.

4-Methylumbelliferyl sulphate, first reported as a fluorogenic substrate for limpet arylsulphatases (Sherman & Stanfield, 1967), has since been used to measure arylsulphatases in various biological fluids (Rinderknecht *et al.*, 1970) and to monitor the purification of soluble and insoluble sulphatases from human nervous tissues (Harinath & Robins, 1971; Perumal & Robins, 1973). Since no studies have yet established whether 4-methylumbelliferyl sulphate could or could not be used as a proper substrate for analysis of arylsulphatases A and B in unfractionated tissues the present investigation was undertaken, using rat liver and human cultured skin fibroblasts as sources of enzymes.

Materials and Methods

Cell lines

Mucopolysaccharidoses (type VI) cells were from the Medical Research Institute (Camden, NJ, U.S.A.). Metachromatic leucodystrophy cell lines were a gift from Dr. H. Goldman (Mutant Cell Repository, deBelle Laboratory of Biochemical Genetics, Montreal Children's Hospital, Montreal, P.Q., Canada).

* To whom reprint requests should be sent.

Enzyme assays

Arylsulphatase A was measured, unless otherwise specificed, with the inhibitory salts of Baum *et al.* (1959) in a buffer consisting of 0.2M-Tris (Sigma Chemical Co. St Louis, MO, U.S.A.)/0.85M-acetic acid adjusted to pH5.0 with 2M-NaOH at 22°C, with either 5.0mM-4-nitrocatechol sulphate (Sigma), or 2.5mM-4-methylumbelliferyl sulphate (Koch–Light Laboratories, Colnbrook, Bucks., U.K.) recrystallized from propane-2-ol/ethyl acetate reactions were stopped by adding 0.5ml of 1M-NaOH or 5ml of 0.1M-2-amino-2-methylpropan-1-ol (G. T. Baker Chemical Co., Philipsburg, NJ, U.S.A.) at pH10.0 respectively. Appropriate controls were performed in all cases. Arylsulphatase B was measured colorimetrically by the method of Baum *et al.* (1959). When 4-methylumbelliferyl sulphate was used in the same buffering conditions, the reaction was stopped by adding 5ml of a 0.1M-2-amino-2-methylpropan-1-ol solution at pH10.0.

Acid β-glycerol phosphatase (EC 3.1.3.2) and β-galactosidase (EC 3.2.1.23) were chosen as lysosomal markers. The former was measured essentially by the method of Gianetto & de Duve (1955). The latter was assayed fluorimetrically by using 0.33mM-4-methylumbelliferyl β-D-galactopyranoside (Koch–Light) as substrate (Robinson, 1964). The reaction was stopped by adding 5ml of 0.1M-2-amino-2-methylpropan-1-ol at pH10.0 and the fluorescence was read with excitation at 360nm and emission at 440nm. Glucose 6-phosphatase (EC 3.1.3.9), which was chosen as the microsomal marker, was measured according to the method of de Duve *et al.* (1955).

Enzyme activity is reported as nmol of substrate cleaved/min, either per gram wet wt. of tissue or per mg of protein measured by the method of Lowry *et al.* (1951) with human serum albumin (Connaught

Table 1. *Comparative sulphatase activity in lysosomal and microsomal-enriched fractions*

The activities were measured as described in the text. The reaction mixtures contained either 5mM-4-nitrocatechol sulphate or 2.5mM-4-methylumbelliferyl sulphate in 0.2M-Tris/0.85M-acetic acid buffer adjusted to pH5.0 with 2M-NaOH or 2.5mM-4-methylumbelliferyl sulphate in 0.2M-sodium phosphate buffer, pH7.5. Results are means±S.D., with the numbers of determinations in parentheses.

	Arylsulphatase A, pH5.0 (nm/min per g wet wt. of tissue)	4-Methylumbelliferyl sulphatase, pH5.0 (nm/min per g wet wt. of tissue)	4-Methylumbelliferyl sulphatase, pH7.5 (nm/min per g wet wt. of tissue)
Lysosomes and mitochondria	366±31 (4)	56.4±1.7 (4)	38.4± 1.0 (4)
Microsomal fraction	49.9±7.4 (4)	63.2±3.5 (4)	172.2±17.1 (4)

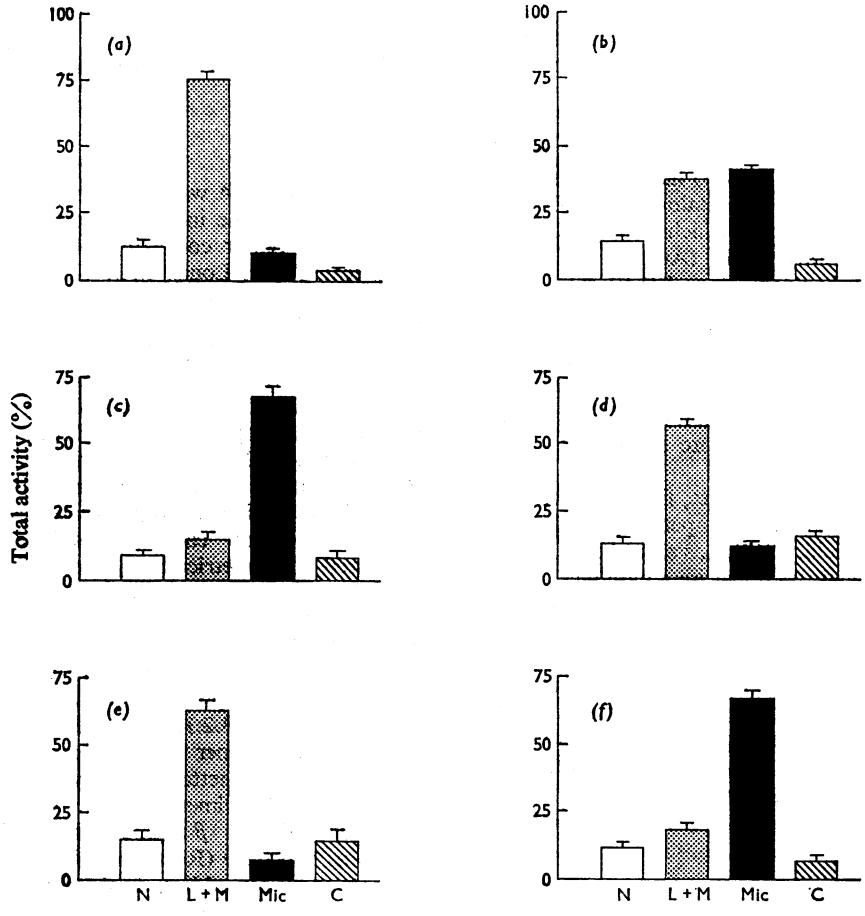

Fig. 1. *Intracellular distribution of rat liver enzymes*

The values are the percentages of the total activity measured in the whole homogenate. (*a*) Arylsulphatase measured with 4-nitrocatechol sulphate in 0.2M-Tris/0.85M-acetic acid adjusted to pH5.0 with 2M-NaOH. (*b*) Arylsulphatase measured with 4-methylumbelliferyl sulphate in 0.2M-Tris/0.85M-acetic acid adjusted to pH5.0 with 2M-NaOH. (*c*) Arylsulphatase measured with 4-methyl umbelliferyl sulphate in 0.2M-sodium phosphate buffer, pH7.5. (*d*) Acid β-glycerol phosphatase measured by the method of Gianetto & de Duve (1955). (*e*) β-D-Galactosidase measured by the method of Robinson (1964). (*f*) Glycose 6-phosphatase measured by the method of de Duve *et al.* (1955). Abbreviations: N, nuclear fraction; L+M, lysosomal+mitochondrial fraction; Mic, microsomal fraction; C, cytosol.

Medical Research Laboratories, Toronto, Ont., Canada) as standard.

Intracellular distribution

Male Black Hooded rats (Canadian Breeding Farms, St. Constant, Que., Canada) weighing 125–150g, deprived of food overnight, were killed by decapitation. Livers were removed at once, blotted, weighed and homogenized in ice-cold 0.25M-sucrose with a Potter–Elvejehm homogenizer fitted with a Teflon pestle. The homogenate was then fractionated by differential centrifugation as described by de Duve *et al.* (1955) except that the mitochondrial and the lysosomal fractions were collected together at an integrated field-time of 196200*g*-min at $r_{av.}$ 7.3cm in the SS-34 rotor of the Sorvall RC2-B centrifuge equilibrated at 4°C. The microsomal fraction was sedimented at an integrated field-time of 3000000*g*-min at $r_{av.}$ 6.4cm in the T-865 rotor of the Sorvall OTD-2 centrifuge equilibrated at 4°C. Each fraction was resuspended in water containing 0.01% Triton X-100 (1ml/g of liver).

Tissue culture

Fibroblasts from control skin and from the skin of three patients [two with metachromatic leucodystrophy and one with mucopolysaccharidosis (type VI)] were cultured in Blake bottles with Eagle's minimum essential medium (NEM; Grand Island Biological Co., Grand Island, NY, U.S.A.) containing 6% (v/v) foetal calf serum (Flow Laboratories, Rockville, MD, U.S.A.) and 6% (v/v) newborn calf serum (Flow Laboratories), and maintained in a O_2+CO_2 (95:5) atmosphere by the method of Hayflick (1965). The medium was changed every 3 days. At confluence the cells were treated with trypsin, washed and spun four times with 0.9% NaCl to remove any trace of medium. Cells were then sonicated at 4°C in water with four bursts of 15s each (12kHz) followed by 15s intervals. The cell suspension, were then spun at 130000*g* ($r_{av.}$ 6.4cm) for 30min at 4°C and the supernatants used for enzyme assays and protein determination.

Results and Discussion

Although 4-methylumbelliferyl sulphate has, in many instances, been used to monitor the purification or the localization of lysosomal arylsulphatases (Hirsch, 1969; Harinath & Robins, 1971; Perumal & Robins, 1973) several points in the present study indicate that it is inadequate to measure these lysosomal enzymes.

Table 1 and Fig. 1 show that arylsulphatase activity and distribution are highly dependent on the substrate used and the pH of incubation. When 4-nitrocatechol sulphate is used in the conditions described by Baum *et al.* (1959) for arylsulphatase A, 75% of the activity is localized in the lysosomal fraction (Fig. 1*a*), following closely the distribution of acid-β-glycerol phosphatase (Fig. 1*d*) and β-D-galactosidase (Fig. 1*e*) as already reported by Viala & Gianetto (1955). However, when 4-methylumbelliferyl sulphate is used, in identical buffering conditions, the activity (4-methylumbelliferyl sulphatase) is almost equally distributed between the microsomal and lysosomal fractions (Fig. 1*b*). Table 1 shows that in these conditions, although the lysosomal sulphatase activity is greatly diminished, the microsomal arylsulphatase activity remains stable. Thus the activities measured with this substrate in unfractionated tissue would equally reflect those of microsomal and lysosomal origins. Upon increasing the incubation pH to 7.0, an increase in activity is noted (Table 1) and we have observed, as did Van Hoof (1972), that the activity concentrates in the microsomal fraction.

Table 2. *Arylsulphatase A and B activities in confluent cultured human skin fibroblasts measured with 4-nitrocatechol sulphate and 4-methylumbelliferyl sulphate*

The activities were measured in the buffering conditions described by Baum *et al.* (1959). Units are nm/min per mg of protein.

	Arylsulphatase A		Arylsulphatase B	
Cell strains	4-Nitrocatechol sulphate	4-Methylumbelliferyl sulphate	4-Nitrocatechol sulphate	4-Methylumbelliferyl sulphate
Control 1	8.56	0.28	4.16	0.54
Control 2	11.52	0.36	3.76	0.47
Control 3	14.02	0.39	7.94	0.74
Control 4	17.48	0.85	8.00	1.00
Control 5	9.80	0.45	14.80	1.51
Mean±S.D.	12.28±3.56	0.47±0.22	7.73±4.43	0.85±0.42
Mucopolysaccharidosis (type VI)	6.80	0.26	<0.10	0.69
Metachromatic leucodystrophy	0.37	0.16	3.18	0.32
	0.46	0.21	9.25	0.25

Milsom *et al.* (1972) have shown that phosphate ions (0.2M, pH7.5) were selectively inhibiting lysosomal arylsulphatases, keeping intact the microsomal activity. In these conditions, like glucose 6-phosphatase (Fig. 1*f*), the majority of 4-methylumbelliferyl sulphatase activity (65%) is localized in the microsomal fraction (Fig. 1*c*). The activity remaining (Table 1) in the lysosomal fraction can be interpreted as being microsomal contamination.

The low hydrolytic rate of 4-methylumbelliferyl sulphate observed in rat tissue, is also demonstrated in unfractionated human skin fibroblasts, therefore compromising the discriminatory power of this assay. Table 2 demonstrates that, in both metachromatic leucodystrophy and mucopolysaccharidosis (type VI), 4-methylumbelliferyl sulphatase activity lies within 1 standard deviation of the mean (obtained from five control cell lines). This contrasts with the severe deficiency of arylsulphatase A and B in metachromic leucodystrophy and mucopolysaccharidosis (type VI) cells respectively when 4-nitrocatechol sulphate is used as substrate.

Therefore these results preclude the use of 4-methylumbelliferyl sulphate to monitor the purification of arylsulphatases from rat liver and to measure arylsulphatase A and B activity in unfractionated human fibroblasts under the conditions of inhibition suggested by Baum *et al.* (1959).

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