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ARYLSULFATASES OF HUMAN TISSUE

STUDIES ON A FORM OF ARYLSULFATASE B FOUND PREDOMINANTLY IN BRAIN

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Summary

The distribution of soluble arylsulfatase (aryl-sulfate sulfohydrolases, EC 3.1.6.1) in human tissues was investigated by DEAE-cellulose chromatography. All tissues examined contained arylsulfatase A and arylsulfatase B. In addition, brain singularly contained significant quantities (15—25% of total arylsulfatase) of a minor anionic arylsulfatase form designated arylsulfatase B_m, whereas only trace amounts of arylsulfatase B_m were found in liver, kidney, testis and placenta. Arylsulfatase B and arylsulfatase B_m had equal activity toward methylumbelliferyl sulfate, nitrocatechol sulfate and a physiological substrate UDP-*N*-acetylgalactosamine 4-sulfate, but both forms were inactive toward the arylsulfatase A substrates cerebroside sulfate and ascorbic acid 2-sulfate. Purified preparations of placental arylsulfatase B, brain arylsulfatase B_m, and urinary arylsulfatase A did not hydrolyze estrone sulfate, dehydroepiandrosterone sulfate or pregnenolone sulfate. The physico-chemical properties of arylsulfatase B and arylsulfatase B_m differed with respect to thermal lability, DEAE-cellulose chromatography, polyacrylamide gel electrophoresis and isoelectric focusing. In the latter technique, utilizing thin polyacrylamide slab gels, the isoelectric point for placental arylsulfatase B was 8.2, while brain arylsulfatase B_m resolved into 3 activity bands with p*I* values 6.8, 7.0 and 7.2. Although the physico-chemical properties differed, arylsulfatase B and arylsulfatase B_m appear to be functionally equivalent as well as generically related.

Introduction

Wortman [1] in 1962 observed that DEAE-cellulose chromatography of beef and rabbit corneal extracts yielded four fractions of arylsulfatases (aryl-sulfate

sulfohydrolase, EC 3.1.6.1), forms a, b, c and d, on the basis of nitrocatechol sulfate and nitrophenyl sulfate hydrolysis. The properties of form d corresponded to those of arylsulfatase A now known to function as cerebroside sulfate sulfohydrolase [2]. Forms a, b and c were presumably different forms of arylsulfatase B. The occurrence of arylsulfatase B in multiple forms has subsequently been reported in beef brain by Bleszynski [3], Bleszynski et al. [4], and Bleszynski and Roy [5]; in beef liver by Allen and Roy [6]; in human placenta by Gniot-Szulzycka [7]; and in human brain, liver, kidney and leukocytes by Harzer et al. [8]. Different isolation conditions and procedures, as well as the use of autolyzed tissues in some studies, have made a comparison of findings of the different investigators somewhat difficult. Likewise, the aforementioned studies were conducted using nonspecific synthetic substrates for arylsulfatase B, rather than a specific physiological substrate, as the physiological function of arylsulfatase B as *N*-acetylgalactosamine 4-sulfate sulfohydrolase [9—11] was only recently established.

Recently we examined the arylsulfatases of cultured human fibroblasts by DEAE-cellulose chromatography [12]. Under the conditions of the ion exchange chromatography, two major fractions, cationic arylsulfatase B and anionic arylsulfatase A, and a minor anionic fraction were found with sulfohydrolase activity toward methylumbelliferyl sulfate. Utilizing this same technique minor anionic arylsulfatases have now been found in significant quantities in extracts from human brain, however, only negligible amounts occur in extracts from liver, kidney, testis and placenta. The minor arylsulfatases are distinguished by having isoelectric points near neutrality, whereas arylsulfatase B has a p*I* greater than 8. Because of these features and the desire not to confuse this group with previously described forms of arylsulfatase B, this enzyme fraction will be referred to as arylsulfatase B_m. Some properties of brain arylsulfatase B_m, including its ability to hydrolyze UDP-*N*-acetylgalactosamine 4-sulfate, were determined.

Experimental

Materials

DEAE-cellulose (Whatman DE-32) was purchased from Reeve Angel (Clifton, N.J.); nitrocatechol sulfate (2-hydroxy-5-nitrophenyl sulfate) from Sigma Chemical Co. (St. Louis, Mo.); polyacrylamide gel reagents from Bio-Rad Laboratories (Richmond, Calif.); carrier ampholytes (Ampholine pH 3—10 and 5—8) from LKB Instruments, Inc. (Rockville, Md.); and [6,7-^{3}H]estrone sulfate (42 Ci/mmol), [7-^{3}H]pregnenolone sulfate (10 Ci/mmol), and [7-^{3}H]-dehydroepiandrosterone sulfate (20 Ci/mmol) from New England Nuclear (Boston, Mass.). 4-Methylumbelliferyl sulfate was purchased from Eastman Kodak Co. (Rochester, N.Y.) and required extensive purification by the method of Rinderknecht et al. [13]. Ascorbic acid 2-sulfate and [^{35}S]ascorbic acid 2-sulfate were gifts from Hoffman-La Roche Inc. (Nutley, N.J.). [^{35}S]Cerebroside sulfate was biosynthesized and isolated as described previously [14]. [^{35}S]UDP-*N*-acetylgalactosamine 4-sulfate was also biosynthesized [15] and isolated [16].

Tissue extracts

Approximately 3 g of human brain, liver, kidney, and testis (obtained at autopsy) or placenta (obtained soon after delivery), stored from 1 month to 4 years at −90°, were homogenized for 2 min with 5 ml 25 mM Tris/chloride, pH 7.5, in a Waring blendor. The homogenate was centrifuged at 12 000 × *g* for 20 min, the precipitated material suspended in 1 ml of the Tris buffer and the mixture recentrifuged. The combined supernatant fluids were dialyzed overnight against 3 l of the buffer. Aliquots were used for enzyme assays, protein determination [17] and ion exchange chromatography.

DEAE-cellulose chromatography

The ion exchange chromatography was carried out as previously described [12]. DEAE-cellulose columns (0.8 × 17 cm) were equilibrated at room temperature with 25 mM Tris/chloride, pH 7.5, and charged with dialyzed extracts. Seven ml of the buffer were pumped through and 26 ml of a linear gradient, 0—0.6 M NaCl in the buffer, were applied. Generally several columns were run in tandem with a common gradient former. The flow rates were adjusted to 12 ml/h and 1 ml fractions were collected from the time the columns were charged.

Polyacrylamide gel electrophoresis

The discontinuous electrophoresis procedure with the triethanolamine/*N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer system at pH 6.8 as described by Orr et al. [18] was used. Enzyme bands were located with methylumbelliferyl sulfate [12].

Isoelectric focusing

Polyacrylamide slab gel isoelectric focusing was carried out as described previously [19] with either a pH 3—10 or a pH 5—8 Ampholine gradient. Briefly, 5% gels were cast, the enzyme in sucrose was layered on top of each lane, and focusing was carried out at 4° C for 4 h with a potential of 700 V during the last hour. Enzyme bands were located with methylumbelliferyl sulfate.

Enzyme assays

The nonspecific methylumbelliferyl sulfate assay system was used to assess the total arylsulfatase activity of tissue extracts and to monitor fractions from the ion exchange chromatography [12]. A unit of activity is defined as the amount of enzyme which catalyzes the hydrolysis of 1 nmol of this substrate per h. Reactive fractions from the chromatography were also examined with the arylsulfatase A and arylsulfatase B specific assay systems of Baum et al [20] with nitrocatechol sulfate. A more convenient but nonspecific assay was also employed for arylsulfatase B fractions freed of arylsulfatase A. Enzyme, 25 µl, and 200 µl of 10 mM nitrocatechol sulfate in 0.5 M sodium acetate, pH 5.8, containing bovine serum albumin (0.2 mg/ml) were incubated for 30 min at 37°. The reaction was terminated with 200 µl 1 M NaOH and the amount of liberated nitrocatechol estimated spectrophotometrically at 515 nm. Assay procedures for cerebroside sulfate [21], ascorbic and 2-sulfate [22], UDP-*N*-

acetylgalactosamine 4-sulfate [11], and steroid sulfate [23] sulfohydrolase activities have been described.

Results

DEAE-cellulose chromatography

DEAE-cellulose chromatography of brain extracts yielded three fractions with arylsulfatase activity as measured by hydrolysis of methylumbelliferyl sulfate (Fig. 1). They corresponded to the fractions derived from chromatography of fibroblast extracts [12]. The unadsorbed fraction has been established as being arylsulfatase B, the low-salt fraction as arylsulfatase B_m and the high-salt fraction as arylsulfatase A. Arylsulfatase B and arylsulfatase B_m fractions were not active with the specific arylsulfatase A assay with nitrocatechol sulfate, but were active with the arylsulfatase B specific assay with this substrate. Arylsulfatase B_m constituted about 15—25% of the total chromatographed arylsulfatase activity in brain extracts. In contrast to brain, chromatography of liver extracts yielded only two fractions: the two major components arylsulfatase A and arylsulfatase B, and essentially no arylsulfatase B_m (Fig. 1). This same pattern was found in liver extracts of several different individuals.

Chromatography of extracts of several brain specimens yielded the same pattern: two major and one minor component. One extract subjected to prior precipitation with 70% ammonium sulfate also gave an essentially identical pattern. In another experiment, the residue from the usual extraction procedure was further extracted with Tris buffer of higher concentration (200 mM). Addi-

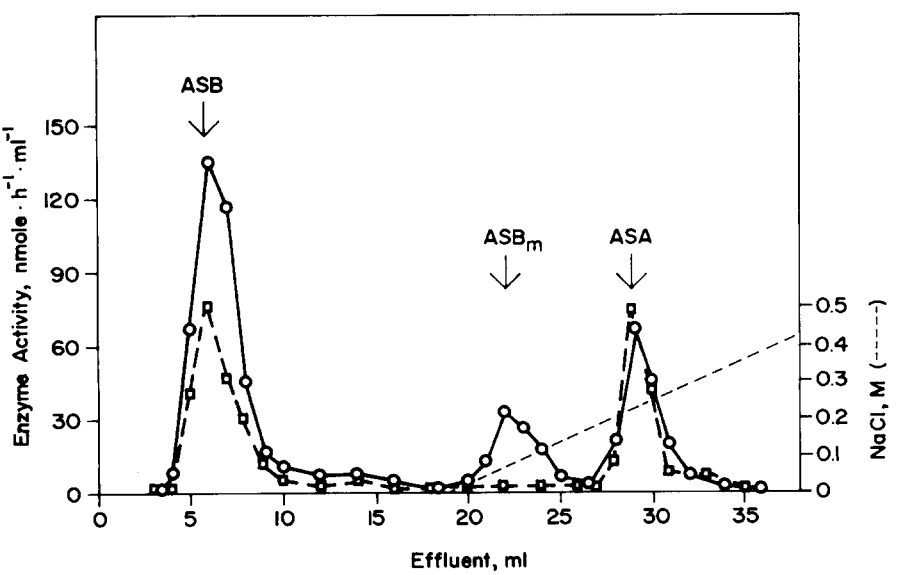

Fig. 1. Arylsulfatase pattern of brain and liver on DEAE-cellulose chromatography. Details of the chromatography are given under Experimental. Dialyzed extracts of brain (○———○, 16.8 mg protein) and liver (□———□, 15 mg protein) were applied to the columns. Activity toward methylumbelliferyl sulfate was determined on 50-μl aliquots of each fraction. Abbreviations: ASB, arylsulfatase B; ASB_m, arylsulfatase B_m; ASA, arylsulfatase A.

tional arylsulfatase activity (about 25%) was extracted and chromatography of this material showed nearly equivalent amounts of enzyme in each fraction. The somewhat increased relative amount of arylsulfatase B_m in the second extract suggests that this form of the enzyme may be membrane-bound [24]. Further study of this phenomenon may provide clues to the origin of arylsulfatase B_m.

A series of tandem chromatographies were carried out on extracts of tissues of a patient with late infantile metachromatic leukodystrophy and a control subject. The brain again contained significant amounts of arylsulfatase B_m (Fig. 2). The arylsulfatase B_m peak is particularly prominent in the metachromatic leukostrophy brain since there is a deficiency of arylsulfatase A. In both cases, arylsulfatase B_m showed activity in the arylsulfatase B specific but not in the arylsulfatase A specific nitrocatechol sulfate assay. Liver and kidney extracts of the control subject gave arylsulfatase A and arylsulfatase B peaks while those of the metachromatic leukodystrophy patient gave only the arylsulfatase B peak with very little if any arylsulfatase B_m (Fig. 2).

Placentas have also been examined by ion exchange chromatography for arylsulfatase B_m. Extracts of either fresh or frozen tissue or of acetone powders yielded only arylsulfatase A and arylsulfatase B and no arylsulfatase B_m. Moreover, unlike liver or kidney even irregularities in the leading arylsulfatase A fractions were absent. Extracts of testis also yielded arylsulfatase A and arylsulfatase B with only the slightest trace of arylsulfatase B_m.

DEAE-cellulose chromatography of a brain extract was carried out on a larger scale. Brain tissue (55 g wet weight) was homogenized as described and the dialyzed extract (410 mg of protein) was placed on a 2.5 × 12.5-cm column. Three peaks of enzyme activity were detected with a slight overlap of the arylsulfatase B_m and arylsulfatase A fractions. The availability of a conductivity meter allowed us to establish that the peak of arylsulfatase B_m occurred at 0.03 M NaCl and the peak of arylsulfatase A near 0.17 M NaCl. (These salt concentrations are lower than those reported earlier [12] for fibroblast extracts where salt concentrations were based on theoretical gradients.) When the arylsulfatase B_m fractions between 0.01 and 0.05 M NaCl were pooled, concentrated, dialyzed, and rechromatographed, the material was recovered at the same NaCl concentration (Fig. 3). The rechromatographed arylsulfatase B_m eluting between 140 and 175 ml was pooled and used for the following studies.

Substrate specificity

The arylsulfatase B_m, pure urinary arylsulfatase A [25], and partially purified placental arylsulfatase B [11] were compared in a variety of sulfatase assays (Table I). The catalytic properties of arylsulfatase B_m and arylsulfatase B were quite similar and distinct from those of arylsulfatase A. It is noteworthy that while there were overlaps with synthetic substrates, high specificity was maintained toward physiological substrates: arylsulfatase B_m and arylsulfatase B were specific toward UDP-*N*-acetylgalactosamine 4-sulfate and arylsulfatase A toward cerebroside sulfate and ascorbic acid 2-sulfate. None of these arylsulfatases were capable of catalyzing the hydrolysis of steroid sulfates. Despite differences in specificity, the pH optima and K_m with methylumbelliferyl sulfate were quite similar for arylsulfatase B_m, arylsulfatase B or arylsulfatase A.

Normal kinetics were exhibited toward this substrate by all the arylsulfatases, but with nitrocatechol sulfate as substrate only arylsulfatase B_m and arylsulfatase B showed normal kinetics.

Sensitivity to Ag^+

The rechromatographed arylsulfatase B_m and the arylsulfatase B and arylsulfatase A recovered from the first chromatography were examined for sensitivity to Ag^+ at 0.02, 0.2 and 2.0 mM. The enzyme preparations were preincubated for 15 min with the metal ion and tested in the methylumbelliferyl sulfate assay. Arylsulfatase B_m and arylsulfatase B were insensitive to all concentra-

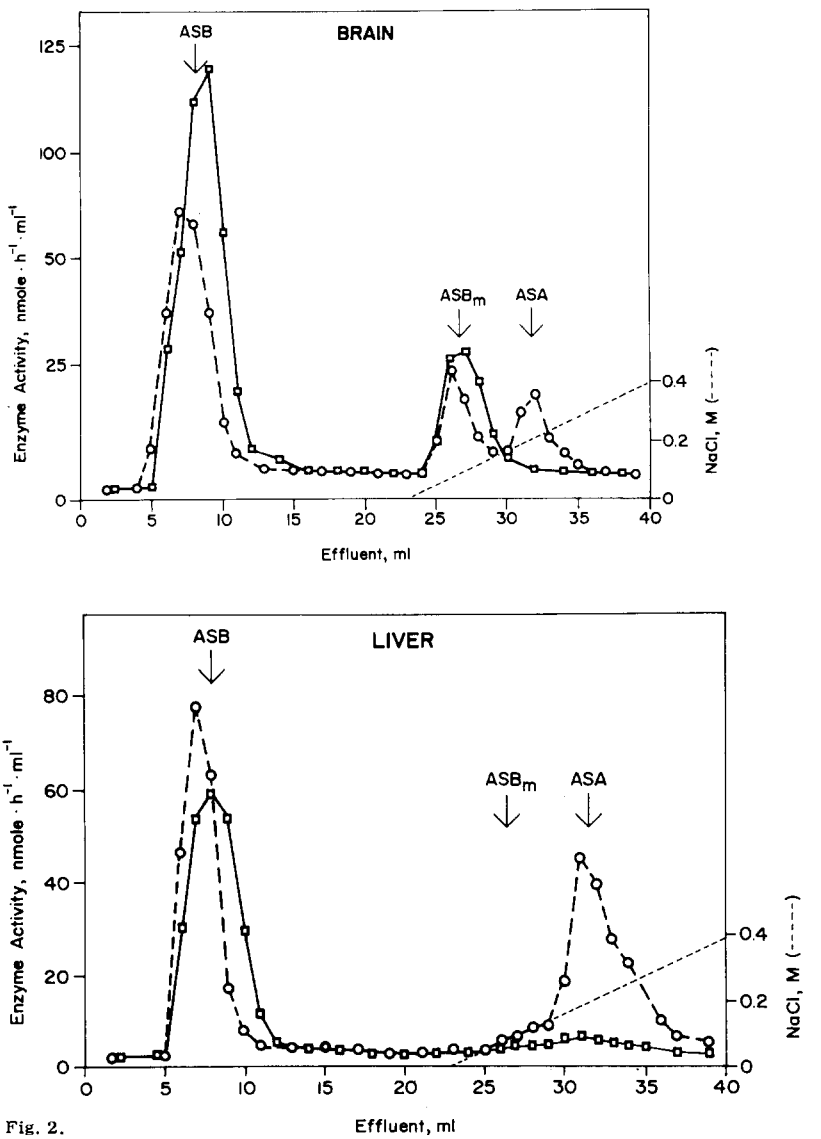

Fig. 2.

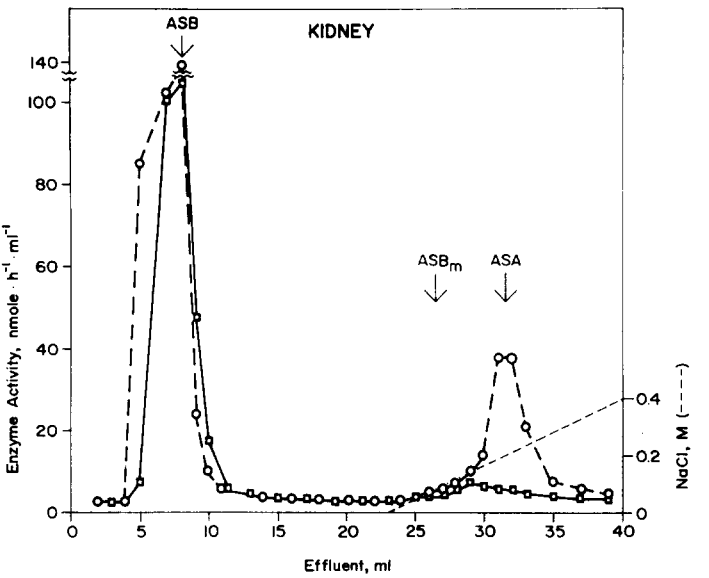

Fig. 2. Arylsulfatase pattern of normal and metachromatic leukodystrophy tissue on DEAE-cellulose chromatography. Details of the chromatography are given under Experimental. Normal, ○———○; metachromatic leukodystrophy, □———□. Brain: normal, 14.9 mg protein; metachromatic leukodystrophy, 15.0 mg protein. Liver: normal, 15.1 mg protein; metachromatic leukodystrophy, 14.8 mg protein. Kidney: normal, 15.0 mg protein; metachromatic leukodystrophy, 15.0 mg protein. Activity toward methylumbelliferyl sulfate was determined on 25-μl aliquots of each fraction. All six columns were developed simultaneously from a common salt gradient. Abbreviations: ASB, arylsulfatase B; ASB_m, arylsulfatase B_m; ASA, arylsulfatase A.

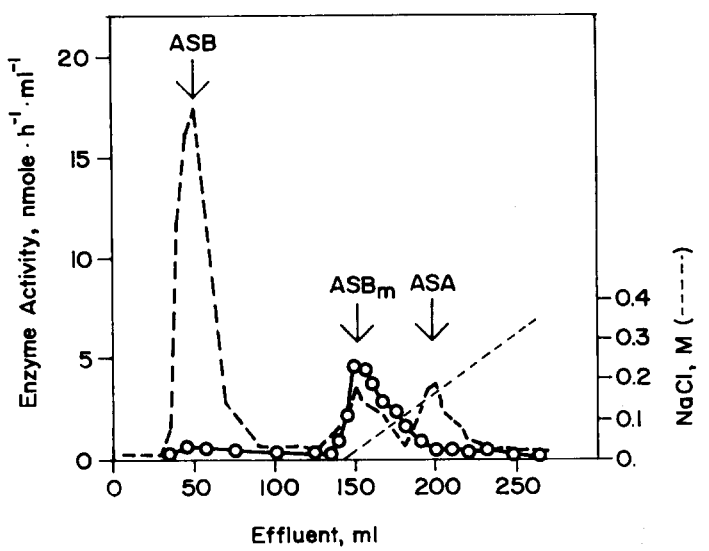

Fig. 3. Rechromatography of brain arylsulfatase B_m on DEAE-cellulose. The column (2.5 X 10 cm) was charged with arylsulfatase B_m, 29 mg protein, obtained from an initial DEAE-cellulose chromatography. Column buffer (80 ml), then 202 ml of a linear gradient, 0 to 0.6 M NaCl in buffer, was applied. The flow rate was maintained at 70 ml/h and 5 ml fractions were collected. Activity toward methylumbelliferyl sulfate was determined on 25 μl aliquots of the fractions. Rechromatographed arylsulfatase B_m, ○———○. Crude dialyzed brain extract, ------, was chromatographed in a parallel column from a common salt gradient. NaCl concentrations were determined by a conductivity meter. Abbreviations: ASB, arylsulfatase B; ASB_m, arylsulfatase B_m; ASA, arylsulfatase A.

tions of Ag^+, but at 0.2 and 2.0 mM the inhibition of arylsulfatase A was greater than 90%.

Thermal stability

The arylsulfatase preparations of the previous paragraph were also exposed to heat treatment at 60° C. All fractions were inactivated by a first-order rate process. Half-inactivation times for arylsulfatase B_m, arylsulfatase B and arylsulfatase A were 7, 27, and 7 min, respectively.

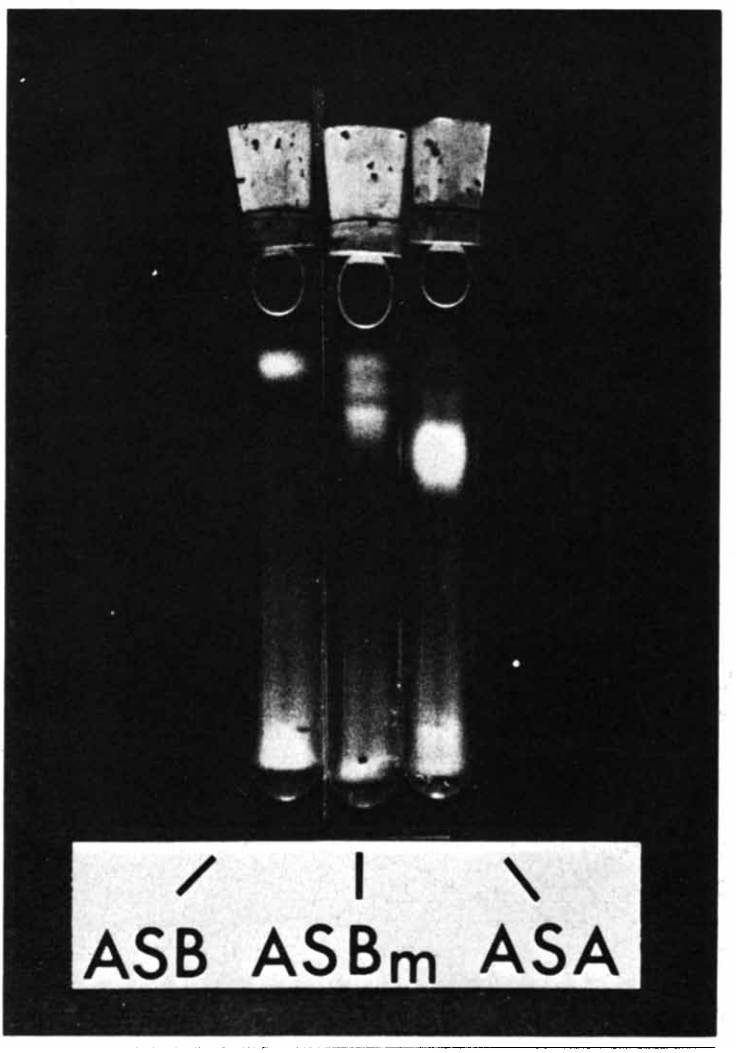

Fig. 4. Polyacrylamide gel electrophoresis of brain arylsulfatase B, arylsulfatase B_m and arylsulfatase A. Gels were charged with 5 units of enzyme and electrophoresed as described previously [25]. Gels were then incubated with methylumbelliferyl sulfate for 20 min at 37° C, the fluorescence enhanced by the addition of 1 M NaOH, and photographed immediately. Abbreviations: ASB, arylsulfatase B; ASB_m, arylsulfatase B_m; ASA, arylsulfatase A.

TABLE I

SUBSTRATE SPECIFICITY OF ARYLSULFATASES

Substrate	Enzyme activity (nmol/h per ml) of arylsulfatase:		
	B [b]	B_m [c]	A [d]
4-Methylumbelliferyl sulfate [a]	244	244	244
4-Nitrocatechol sulfate			
Nonspecific	2630	2840	1300
Arylsulfatase B specific	1220	1250	70
Arylsulfatase A specific	55	45	3880
UDP-*N*-Acetylgalactosamine 4-sulfate	16	33	None
Cerebroside sulfate	None	None	89
Ascorbic acid 2-sulfate	None	None	1070
Estrone sulfate	None	None	None
Dehydroepiandrosterone sulfate	None	None	None
Pregnenolone sulfate	None	None	None

[a] Each enzyme solution was adjusted to equivalent units toward this substrate.
[b] Purified placental enzyme.
[c] Rechromatographed brain enzyme.
[d] Pure urinary enzyme.

Electrophoresis

The migration of arylsulfatase B_m, arylsulfatase B and arylsulfatase A on discontinuous polyacrylamide gel electrophoresis is shown in Fig. 4. Under the present conditions (pH 6.8), arylsulfatase B remained at the cathode while aryl-

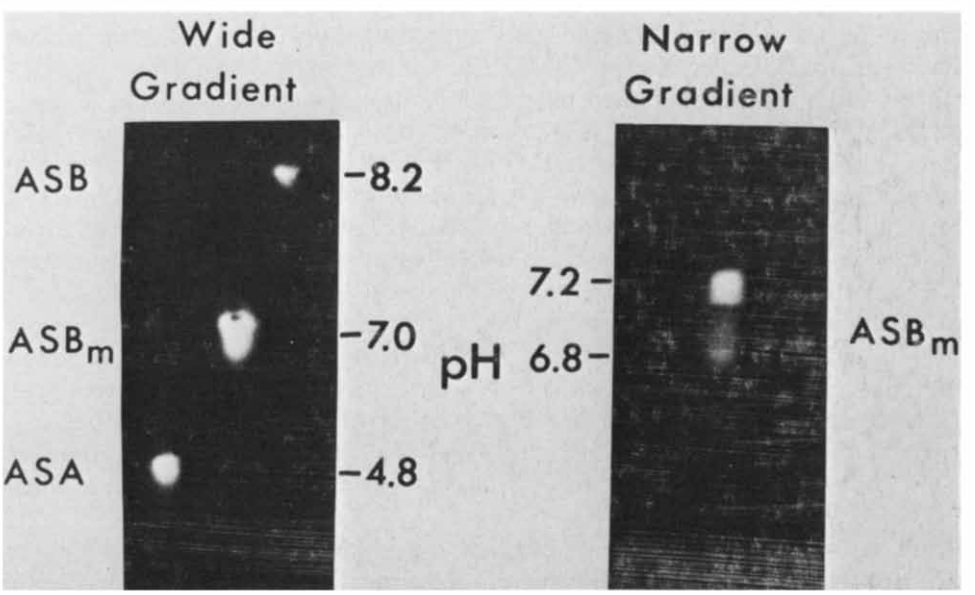

Fig. 5. Slab gel isoelectric focusing of placental arylsulfatase B, brain arylsulfatase B_m and urinary arylsulfatase A. Details of the focusing are given under Experimental. Wide gradient (Ampholine, pH 3—10), enzyme per lane: arylsulfatase B, 13 units; arylsulfatase B_m, 13 units; arylsulfatase A, 5.3 units. Narrow gradient (Ampholine, pH 5—8), arylsulfatase B_m, 8 units. The fluorescent staining procedure was identical to that used for Fig. 4. Abbreviations: ASB, arylsulfatase B; ASB_m, arylsulfatase B_m; ASA, arylsulfatase A.

sulfatase A migrated toward the anode. Arylsulfatase B_m also migrated toward the anode, but its mobility was lower than that of arylsulfatase A. The brain arylsulfatase B_m, like the fibroblast arylsulfatase B_m [12], did not yield crisp bands. Instead, there were two or three diffuse bands.

Isoelectric focusing

Wide pH gradient (pH 3—10) slab gel isoelectric focusing of the rechromatographed brain arylsulfatase B_m, urinary arylsulfatase A [25], and placental arylsulfatase B [11] showed enzyme bands with widely spaced p*I* values (Fig. 5). Arylsulfatase A focused at pH 4.8; arylsulfatase B focused at pH 8.2; and arylsulfatase B_m focused around pH 7.0 in several contiguous bands. Isoelectric focusing of arylsulfatase B_m in a narrow pH gradient (pH 5—8) yielded three discrete bands with p*I* values of about 6.8, 7.0 and 7.2 (Fig. 5).

Discussion

As a result of genetic disease studies a number of different sulfatases are now known to occur in human tissues [26]. Several of these have not been associated with sulfatase fractions which can be detected with chromogenic or fluorogenic assay substrates. The finding of a minor arylsulfatase activity in human fibroblasts with ion exchange properties distinct from either arylsulfatase A or arylsulfatase B raises the possibility that this might represent a functionally distinct enzyme [12]. Conversely, the observation that the activity of this component was decreased in Maroteaux-Lamy fibroblasts indicates that the minor component may be an alternative form of arylsulfatase B [27]. Since cultured fibroblasts were an inadequate source of arylsulfatase B_m for further characterization, human tissues were evaluated for the presence of this enzyme form. Of those tissues examined only brain contained arylsulfatase B_m in more than trace amounts. In brain, this fraction constituted 15—25% of the total soluble arylsulfatases as compared to the 5—10% we had previously found in cultured fibroblasts [12]. There were trace amounts of arylsulfatase B_m in liver, kidney and testis, while placenta appeared to be devoid of this sulfatase form. Human brain, therefore, was selected as the source for preparing arylsulfatase B_m, free from arylsulfatase A and arylsulfatase B, in quantities adequate for a more detailed enzymatic characterization.

Fractionation of the brain extract on DEAE-cellulose with a salt gradient elution provided a material free of the typical forms of arylsulfatase A and arylsulfatase B as judged by rechromatography on DEAE or by isoelectric focusing. Narrow gradient isoelectric focusing revealed that the arylsulfatase B_m fraction was polydisperse, consisting of three closely spaced bands with p*I* values near pH 7.

Brain arylsulfatase B_m was catalytically similar to arylsulfatase B with synthetic substrates as indicated by normal reaction kinetics with nitrocatechol sulfate, resistance to inhibition by Ag^+, activity in the arylsulfatase B specific and inactivity in the arylsulfatase A specific nitrocatechol sulfate assays. The high reactivity of arylsulfatase B_m toward an apparently specific physiological substrate of arylsulfatase B, UDP-*N*-acetylgalactosamine 4-sulfate, and the complete inactivity toward cerebroside sulfate, ascorbic acid 2-sulfate, estrone

sulfate, dehydroepiandrosterone sulfate, and pregnenolone sulfate led to the conclusion that arylsulfatase B_m is functionally related to arylsulfatase B but not to arylsulfatase A or arylsulfatase C. The previous observation that arylsulfatase B_m in Maroteaux-Lamy syndrome fibroblasts was deficient in proportion to the arylsulfatase B deficiency [27] supports the concept that these two enzyme species are generically related. While it is still possible that one of the subcomponents of the arylsulfatase B_m fraction could represent a distinct sulfatase, the close correspondence of arylsulfatase B and arylsulfatase B_m in relative activities for different substrates makes this improbable for any of the major subspecies.

The catalytic identity notwithstanding, arylsulfatase B_m is physicochemically distinct from arylsulfatase B as indicated by differences in affinity for ion exchange resins, mobility on gel electrophoresis, isoelectric point, and thermal susceptibility. At present we have no information on the factors responsible for these differences, but it seems likely that they represent variations in post-synthetic alteration of a common primary gene product. The large difference in p*I* does indicate a rather substantial change in ionic constituents. Further, the high proportion of arylsulfatase B_m in brain suggests a special role for this enzyme form, but any understanding of its functional significance will require more extensive studies.

Other workers have described a variety of arylsulfatase B-like subfractions from human sources, but it is difficult to assess their relationship to the material reported herein. Gniot-Szulzycka [7] reported that human placenta arylsulfatase B was resolved into three components, one major and two minor, by CM-cellulose chromatography. The p*I* of the major component was 8.1 and those of the minor components 7.9 and 7.6. Under the conditions of our DEAE-cellulose chromatography all of these components would have appeared in the unadsorbed fraction (arylsulfatase B). However, our placental arylsulfatase B preparation contained only one band of activity on isoelectric focusing (p*I* 8.2). It is possible that the extensive autolysis of placenta by Gniot-Szulzycka may have led to the formation of the minor components. In our studies, human placenta did not appear to contain any arylsulfatase B_m or other minor components.

Harinath and Robbins [28] also subjected human brain extracts to DEAE-cellulose chromatography and they observed a very minor peak (less than 1% of the total arylsulfatase activity) at about the NaCl concentration where arylsulfatase B_m is eluted. A seemingly minor difference in the concentration of the chromatography buffer (their buffer, 50 mM; our buffer, 25 mM) probably accounts for their 1% compared to our 15—25% arylsulfatase B_m. We had observed earlier that with 50 mM buffer most of the arylsulfatase B_m is not adsorbed by the resin and is admixed with arylsulfatase B in the unadsorbed fraction [12].

In one other study of human tissues, Harzer et al. [8] subjected brain, liver, kidney and leukocytes to column isoelectric focusing. They obtained two peaks at p*I* values of 8.7 and 8.2 with catalytic properties of arylsulfatase B, and in most tissues the amounts of the p*I* 8.7 component greatly exceeded that of the p*I* 8.2 component. In brain, the p*I* 8.2 component was predominant, but there was also other material in the neutral region of the pH gradient. No comment

was made by the authors on this latter arylsulfatase activity, but it could be similar to the arylsulfatase B_m which we see in brain extracts.

In conclusion, brain was found to be particularly rich in arylsulfatase B_m, an arylsulfatase fraction with p*I* values near pH 7. Arylsulfatase B_m and arylsulfatase B appear to be enzymatically indistinguishable and probably act on the same physiological substrates. The concept that arylsulfatase B_m is derived from arylsulfatase B as a result of post-ribosomal modifications is attractive. The uniquely high proportion of this fraction in brain raises the possibility of a special function in neural tissue. However, further studies will be required to establish the significance and role of arylsulfatase B_m.

Acknowledgements

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