Biochimica et Biophysica Acta, 445 (1976) 661—671
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

BBA 67931

MICROHETEROGENEITY OF ARYLSULFATASE A FROM HUMAN TISSUES

RICHARD L. STEVENS, ARVAN L. FLUHARTY, AUDREY R. KILLGROVE and HAYATO KIHARA

Neuropsychiatric Institute-Pacific State Hospital Research Group, Pomona, Calif. 91766 (U.S.A.)

(Received February 24th, 1976)
(Revised manuscript received June 21st, 1976)

Summary

Human arylsulfatase A (cerebroside-3-sulfate 3-sulfohydrolase, EC 3.1.6.8) exhibited microheterogeneity on isoelectric focusing in polyacrylamide gels. Pure urinary enzyme gave 3 bands of activity with p*I* values of 4.7, 4.8 and 4.9, whereas purified liver enzyme yielded six equally spaced bands from p*I* 4.4 to 4.9. Detection of enzyme in the gel was made by either methylumbelliferyl sulfate or nitrocatechol sulfate. Crude enzyme preparations from human liver, kidney, placenta, brain and testis showed the six-banded pattern with varying amounts of activity in the different bands. The banding pattern of cultured human fibroblast extracts was distinctive: in addition to activity in the area of Bands 1—6 a sharp band at p*I* 5.1 was observed with both enzyme stains. This latter band was also present in metachromatic leukodystrophy fibroblast extracts. However, in this case the band did not appear when the specific arylsulfatase A stain was used. Enzyme Bands 1, 2 and 3 from urine were isolated by extraction of the gel. The three bands refocused in their initial positions; showed nearly identical enzymatic activities toward methylumbelliferyl sulfate, nitrocatechol sulfate, cerebroside sulfate and ascorbic acid 2-sulfate; and demonstrated equivalent immunological competence by antibody titration. The banding pattern of urinary arylsulfatase A was unchanged with neuraminidase treatment, whereas Bands 4—6 of the liver enzyme were converted to Bands 1—3 by this treatment. It appears that Bands 4—6 are due to sialylation of arylsulfatase A but that Bands 1—3 are probably due to some other type of post-ribosomal protein modification.

Introduction

Cerebroside sulfate sulfohydrolase (cerebroside-3-sulfate 3-sulfohydrolase, EC 3.1.6.8, trivially known as arylsulfatase A or sulfatase A) has been purified

essentially to homogeneity from pooled human urine [1]. This enzyme preparation yielded a single band on polyacrylamide gel electrophoresis in the absence or presence of sodium dodecyl sulfate and on wide pH gradient gel isoelectric focusing. However, recent reexamination by narrow pH gradient isoelectric focusing revealed 3 discrete bands with enzyme activity. Similar examination of purified human liver enzyme yielded six bands: three corresponding to those obtained with the urine enzyme and three additional bands. To determine whether the fewer number of bands in the urine enzyme might be the result of selection during purification, crude preparations were examined. The same three bands and only those bands were present. It thus appeared that there was heterogeneity in "pure" enzyme preparations and also microheterogeneity between enzyme from different tissues. The examination by isoelectric focusing was extended to include enzyme derived from kidney, placenta, brain, testis and cultured fibroblasts. This communication is concerned with the observed tissue-specific microheterogeneity of human arylsulfatase A and some properties of the three forms derived from the urine enzyme.

Experimental

Materials. Carrier ampholytes (Ampholine, pH 3—6) were purchased from LKB Instruments, Inc. (Rockville, Md.); polyacrylamide gel reagents and DEAE-cellulose from Bio-Rad Laboratories (Richmond, Calif.); CM-cellulose (Whatman CM-32) from Reeve Angel (Clifton, N.J.); nitrocatechol sulfate (2-hydroxy-5-nitrophenyl sulfate) from Sigma Chemical Co. (St. Louis, Mo.); and *Clostridium perfringens* neuraminidase (24 units/mg, merthiolate free) from Worthington Biochemical Corp. (Freehold, N.J.). ^{35}S-Labeled ascorbic acid 2-sulfate was provided by the Department of Chemical Research, Hoffman-La Roche Inc. (Nutley, N.J.). ^{35}S-Labeled cerebroside sulfate was biosynthesized and isolated as previously described [2]. 4-Methylumbelliferyl sulfate was purchased from Eastman Kodak Co. (Rochester, N.Y.) and required extensive purification by the method of Rinderknecht et al. [3]. Rabbit anti-arylsulfatase A serum [1] and goat anti-rabbit serum (Hyland; Costa Mesa, Calif.) were purified by DEAE-cellulose chromatography.

Polyacrylamide slab gel isoelectric focusing. A Hoefer dual vertical slab gel electrophoresis cell (Bio-Rad Model 220) with an LKB power supply (Model 3371 D) was used. The gel mixture, adapted from that of Finlayson and Chrambach [4], contained: acrylamide, 5%; *N,N'*-methylenebisacrylamide, 0.2%; carrier ampholytes, pH 3—6, 2%; glycerol, 5%; *N,N,N',N'*-tetramethylethylenediamine, 0.1%; ammonium persulfate, 0.015% and riboflavin, 0.0005%. The mixture was poured into a slab mold (200 × 200 × 0.75 mm) fitted with a 20-tooth comb and polymerized under fluorescent light at room temperature for 1 h. 10 µl of sucrose/ampholyte (5% : 2%) solution were placed in each comb sample well and underlayed with enzyme samples (10—20 µl) in sucrose/ampholyte (10% : 2%) solution. Typically, the experiments were carried out with 2—3 units of enzyme per lane, but satisfactory resolution was obtained with 20—30 units per lane and as little as 0.05 units could be visualized. A unit of enzyme activity is defined as 1 µmol of nitrocatechol sulfate hydrolyzed/h under our standard conditions [1]. The three center lanes were usually left vacant for pH determination. The lower (anodal) chamber was filled with 10

mM phosphoric acid and upper (cathodal) chamber with 20 mM NaOH. The coolant chamber was filled with cold (4°C) water and the focusing was carried out at 3—3.5 W by increasing the voltage stepwise from 200 to 700 V. The voltage was maintained at 700 V for the final 0.5—1 h giving a total of 3—4 h for the experimental procedure. For reasons yet unclear, incompletely focused runs were occasionally obtained. Therefore, purified urine arylsulfatase A was included in all experiments and its pattern served to indicate whether proper focusing had been achieved.

pH determination and enzyme localization. The vacant center lanes were sliced at 0.5 cm intervals, eluted overnight in boiled water and the pH determined.

Enzyme bands were located by overlaying gels with a methylumbelliferyl sulfate solution (10 mM in 0.5 M sodium acetate, pH 5.5). Liberated 4-methylumbelliferone could be seen under long-wavelength ultraviolet light in about 10 min. When less than 0.5 unit of enzyme was used, the reaction with the substrate was carried out at 37°C for 15 min and the fluorescence of the product was intensified by treating the gel with 1 M NaOH. Since the fluorogenic substrate reacts equally well with arylsulfatases A and B, duplicate gels were treated with nitrocatechol sulfate as used in the arylsulfatase A specific assay [5]. The substrate (10 mM in 0.5 M sodium acetate, pH 5.0, with 10% NaCl and 0.1 mM sodium pyrophosphate) was poured on the gel, incubated at 37°C for 10—20 min, then drained off. The red enzyme stain was developed by the addition of 1 M NaOH.

Enzyme preparations. Acetone powders of tissues (liver, kidney, brain and testis obtained at autopsy or placenta soon after delivery, stored at —90°C) were prepared by homogenizing 3 g of sliced frozen tissue with 4 vol. cold acetone in a Waring blender and filtering the mixture on a Buchner funnel. The residue was washed with 4 vol. acetone/ether (1 : 1) and 4 vol. ether, then air dried. The powder (about 500 mg) was extracted with 4 ml 25 mM Tris · HCl, pH 7.5, for 30 min and centrifuged for 20 min at 10 000 × *g*. The pellet was reextracted with 2 ml buffer and the combined extracts were dialyzed overnight against 4 l of this same buffer. The dialyzed extracts were passed through a CM-cellulose column equilibrated with 25 mM Tris · HCl, pH 7.5. The fraction not adsorbed by the resin was concentrated about 5-fold by ultrafiltration (Amicon UM-20 membrane). Since brain extracts contained less arylsulfatase A than other tissues, an additional DEAE-cellulose chromatography [6] was required.

Pure urine arylsulfatase A was prepared as previously described [1] and purified liver enzyme was provided by Drs. R.K. Draper and J. Edmond [7].

Enzyme samples from human fibroblasts were obtained from cultured cells that were grown, harvested and extracted as previously described [6].

Isolation of focused urine enzymes. Purified urine arylsulfatase A (80 units) was applied to all lanes of a slab gel and subjected to isoelectric focusing by the standard procedure. Enzyme bands located with methylumbelliferyl sulfate were cut out and extracted from the gel overnight at 4°C with 25 mM Tris · HCl, pH 7.5, containing 0.1 M NaCl and ribonuclease (1 mg/ml). The ribonuclease was included to avoid inactivation of arylsulfatase A in dilute solutions [1]. Extracts were concentrated by ultrafiltration and dialyzed against 25 mM

Tris · HCl, pH 7.5. Aliquots were used for refocusing, enzyme assays, and antibody titration.

Enzyme assays. Arylsulfatase A activity of material to be focused was determined in the usual manner with nitrocatechol sulfate [1]. Material recovered after resolution by isoelectric focusing into three bands was assessed for activity toward methylumbelliferyl sulfate [1], cerebroside sulfate [8], and ascorbic acid 2-sulfate [9] as well as nitrocatechol sulfate.

Immunotitration. 25 µl of antiserum (diluted to precipitate 0.09 unit of arylsulfatase A) was mixed with 25 µl of test enzyme solution (0.07—0.23 units) diluted in 25 mM Tris · HCl, pH 7.5, containing bovine serum albumin (1 mg/ml). After incubation at 37°C for 30 min, 10 µl of goat anti-rabbit serum were added and reincubated for 30 min. Tubes were placed in an ice bath for 1 h, then centrifuged at 12 000 × *g* for 1 min. The amount of enzyme in excess of antibody was determined by measuring the arylsulfatase A activity toward nitrocatechol sulfate in a 25 µl aliquot of the supernatant solution. The control series carried out in parallel contained enzyme and goat anti-rabbit serum, but no anti-arylsulfatase A serum. The difference between the control and test series extrapolated to zero enzyme activity in the supernatant solution represents the capacity of the antibody sample to bind the test enzyme.

Neuraminidase treatment. Urine arylsulfatase A (50 units) was reacted with 0.05 unit neuraminidase in 0.1 M potassium acetate buffer, pH 4.5, in a total volume of 100 µl at 37°C for 16 h. Liver arylsulfatase A (13 units) was treated with 0.03 unit neuraminidase in 60 µl buffer at 37°C for 16 h. The incubation was terminated by placing on ice. Then aliquots were taken for enzyme activity measurements and for isoelectric focusing.

Results

Pure urinary arylsulfatase A was resolved by isoelectric focusing into three bands of activity which appeared to be present at equivalent concentrations. The bands were uniformly spaced and the p*I* values were about 4.9, 4.8 and 4.7 for the arbitrarily designated Bands 1, 2 and 3. In contrast, a highly purified preparation of arylsulfatase A from liver yielded six bands: three corresponding to those obtained from the urine enzyme and three others with p*I* values of

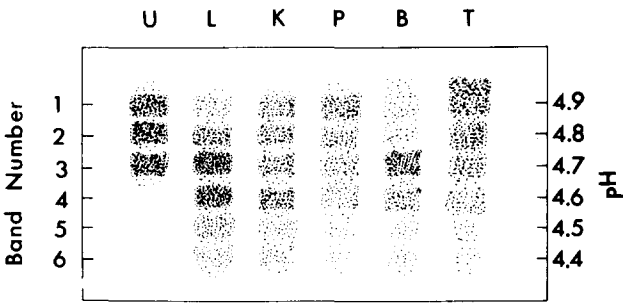

Fig. 1. The isoelectric focusing banding patterns of arylsulfatase A derived from different tissues. U, urine; L, liver; K, kidney; P, placenta; B, brain; and T, testis. Enzyme bands were located with methylumbelliferyl sulfate and nitrocatechol sulfate.

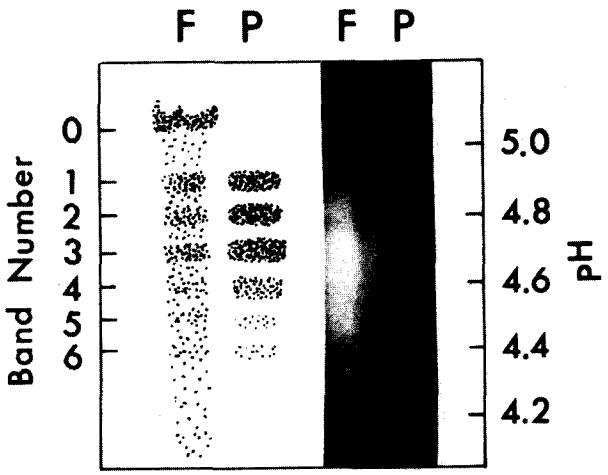

Fig. 2. Isoelectric focusing of arylsulfatase A from fibroblasts and placenta. F, 0.05 unit fibroblast enzyme, and P, 0.12 unit placenta enzyme.

about 4.6, 4.5 and 4.4 for Bands 4, 5 and 6, respectively. The uniform spacing prevailed over all six bands. The patterns with crude enzyme preparations from urine and liver were identical to the pattern with pure enzymes.

Isoelectric focusing of crude enzyme preparations from liver, kidney, placenta, brain and testis yielded multiple bands of activity which appeared to correspond to Bands 1—6 derived from purified liver arylsulfatase A (Fig. 1). However, the relative amounts of enzyme in each band were different for each tissue. In the preparation from liver Bands 2, 3 and 4 were present in greater amounts than Bands 1, 5 and 6; in kidney Bands 1—4 were the major bands; in placenta Bands 1, 2 and 3 dominated with somewhat less in Band 4 and trace amounts in Bands 5 and 6; in brain Bands 3 and 4 were the major bands while the others were weak and diffuse; in testis Bands 1—4 dominated and Band 1 was broader than usual. The figure is a summation of many experiments carried out with different amounts of enzyme. The characteristic banding pattern of enzyme from different tissue was quite reproducible. Tissue from several subjects gave similar patterns, but polymorphism cannot be completely precluded because of the limited number of samples examined.

Isoelectric focusing of arylsulfatase A preparations from cultured fibroblasts yielded patterns which were unlike those previously observed (Fig. 2). With all gels, fluorescence in bands with high amounts of enzyme is visible soon after application of the substrate, but bands with less enzyme are not visible for a number of minutes. By then the fluorescence of the former bands has spread and overlapped so it is quite difficult to reproduce photographically all the features observed visibly with time. Arylsulfatase A from fibroblast extracts was especially troublesome in this respect, but Fig. 2 gives our visual impression of a number of experiments. Bands 1, 2 and 3 were fairly well defined, but activity in the region of Bands 4—6 was ill defined and diffuse. In addition, there was a sharp band before Band 1 which we have designated Band 0. Band 0 was unique not only by its presence, but also by its irregular shape and

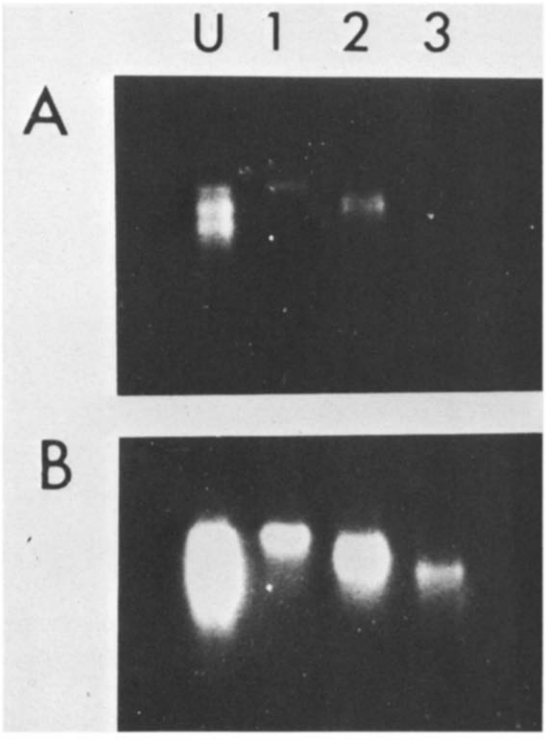

Fig. 3. Refocusing of Bands 1, 2 and 3 from urine arylsulfatase A. U, 1.0 unit urine enzyme; 1, 0.3 unit Band 1; 2, 0.5 unit Band 2; and 3, 0.2 unit Band 3. A, focused gel incubated with methylumbelliferyl sulfate for 10 min at 37°C; B, A treated with 1 M NaOH.

departure from the uniform spacing seen with Bands 1—6. Its p*I* was about 5.1. Band 0, like the other bands, reacted with the arylsulfatase A specific nitrocatechol sulfate reagent as well as with the nonspecific fluorogenic reagent. This same arylsulfatase banding pattern was observed with crude fibroblast extracts before and after dialysis. Likewise, enzyme prepared by CM-cellulose or DEAE-cellulose chromatography of acetone powder extracts exhibited the same pattern. Preparations from fibroblasts derived from patients with Maroteaux-Lamy syndrome gave patterns indistinguishable from patterns with normal fibroblasts and all bands reacted with nitrocatechol sulfate. Preparations from fibroblasts derived from patients with metachromatic leukodystrophy yielded only Band 0 at about the same intensity as the band in normal fibroblasts; in these instances, however, Band 0 reacted only with methylumbelliferyl sulfate and not with the nitrocatechol sulfate reagent which specifically detects arylsulfatase A. The cerebroside sulfate sulfohydrolase activity was not tested.

To determine whether the individual enzyme bands were stable molecular species, material in Bands 1, 2 and 3 from urine arylsulfatase A was isolated and refocused. Each band focused true, i.e. material from Band 1 migrated with a p*I* of only Band 1; from Band 2 as only Band 2; and from Band 3 as only Band 3 (Fig. 3). Examination of the fluorescent stained gel before and after alkali treatment illustrates the separation of Bands 1, 2 and 3 and shows the

TABLE I

ENZYME ACTIVITIES OF THE THREE FORMS OF ISOELECTRIC FOCUSED URINE ARYLSULFATASE A

Substrate	Activity units (μmol/h)		
	Band 1	Band 2	Band 3
Nitrocatechol sulfate	5.1	5.6	5.3
Methylumbelliferyl sulfate	0.4	0.4	0.4
Cerebroside sulfate	0.15	0.2	0.1
Ascorbic acid 2-sulfate	1.5	1.6	1.5

problem of obtaining all the useful information on one picture. The alkali treatment enhances the detection of low activity bands but at the same time masks band separation due to rapid product diffusion and enhanced enzyme detection at the periphery of strong bands. Thus, it appeared that the bands were neither equilibrium mixtures nor an artifact of the isoelectric focusing procedure.

The enzymes isolated from Bands 1, 2 and 3 were examined for activity toward two synthetic (nitrocatechol sulfate and methylumbelliferyl sulfate) and two natural (cerebroside sulfate and ascorbic acid 2-sulfate) substrates of arylsulfatase A (Table I). The ratios of activities toward the various substrates were quite similar for enzyme from each band. Thus, the enzymatic competence of the material in each band was essentially identical. The relative amounts of enzyme in each band appeared to be nearly equivalent, but since the overall recovery was only 20% this observation must be considered tentative.

The enzymes isolated from bands 1, 2 and 3 were examined for quantitative antibody reactivity. The amount of antibody bound per unit enzyme activity was identical for enzyme from all three bands (Fig. 4) and also equal to that of the unfocused pure enzyme. Therefore, the immunological competence was equivalent for the three enzyme forms.

Neuraminidase-treated urine arylsulfatase A retained both full reactivity with nitrocatechol sulfate and its characteristic three-banded pattern on isoelectric focusing. All three bands were of equal intensity before and after the treatment. However, the six-banded isoelectric focusing pattern of liver arylsulfatase

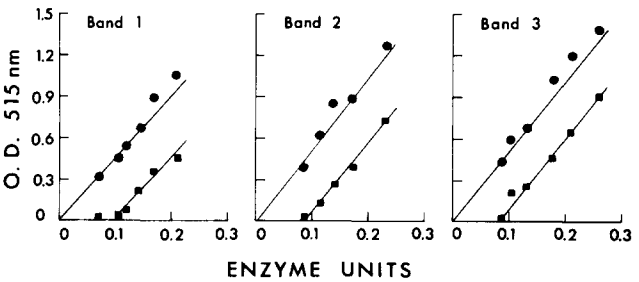

Fig. 4. Enzyme-antibody titration of Bands 1, 2 and 3 from urine arylsulfatase A. Enzyme activity remaining in solution after precipitation with antibody was determined with nitrocatechol sulfate and expressed as A_{515} nm. The experimental procedure is described in the text. ■, complete; ●, minus anti-arylsulfatase A serum.

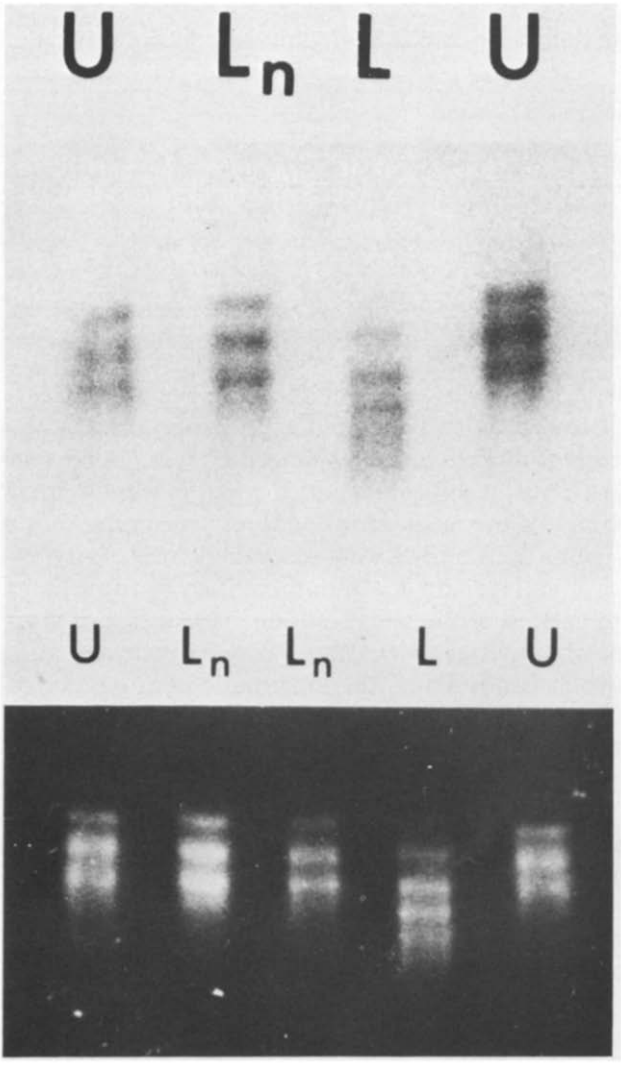

Fig. 5. The effect of neuraminidase treatment on the isoelectric focusing banding pattern of liver arylsulfatase A. U, 1.3 units of urine enzyme; K, 1.1 units of liver enzyme; and L_n, 1.2 units of liver enzyme treated with neuraminidase. Top, gel incubated with nitrocatechol sulfate for 10 min at 37°C then with 1 M NaOH. Bottom, similar gel incubated with methylumbelliferyl sulfate for 20 min at room temperature.

A was changed by the treatment (Fig. 5). The neuraminidase-treated enzyme no longer contained Bands 4—6. Since no enzyme activity was lost in this treatment Bands 4—6 apparently have been converted to the less electronegative Bands 1—3. This shift is most dramatic with Band 1 in which a faint activity stain in the untreated enzyme is now supplanted by a band equal in intensity to that of Bands 2 and 3. The six-banded pattern was retained by the neuraminidase-less control, otherwise treated identically.

Discussion

An increasing number of lysosomal hydrolases have been discovered to exist in multiple forms (e.g., refs. 10—12). The present observations indicate that arylsulfatase A, long believed to be unique in this respect, is no exception. A second minor form of arylsulfatase A from human urine had been described, but it was an unstable form derived in vitro which reverted spontaneously to the major species [13]. In contrast, the present multiple forms of arylsulfatase A appear to be stable molecular entities and, like other lysosomal hydrolases, there is microheterogeneity in "pure" enzyme preparations. In addition the pattern of this microheterogeneity varies with enzyme derived from different tissues.

We, as well as others, have previously subjected arylsulfatase A to isoelectric focusing and failed to note the existence of multiple forms [13—16]. In part, this can be attributed to the use of wide pH gradients which did not allow resolution of material with very close p*I* values. After the present discovery, preparative isoelectric focusing with a narrow pH gradient was carried out on placental arylsulfatase A. The elution profile was flat and broad without discrete peaks. Leading and tailing fractions analyzed by the slab gel isoelectric focusing system showed enrichment of Bands 3 and 1, respectively, but resolution was incomplete (unpublished observations). Complete resolution was achieved with slab gels and elution of individual components provided us with sufficient amounts of material for the present studies. However, the low capacity and recovery indicates that improved techniques would be desirable for conducting further probes.

In the survey of enzyme from different tissues we initially tried to employ crude extracts to avoid possible inadvertant selective purification. However, extracts of acetone powders could not be used in most cases because it was difficult to apply sufficient enzyme units to gels. Moreover, excessive extraneous proteins precipitated in the sample wells and occluded much of the enzyme. For most tissues a single ion exchange chromatography step provided material amenable to isoelectric focusing. It is interesting that while such procedures eliminate only cationic material leaving all material with p*I* values similar to the enzyme, focusing resolution was much improved. The nature and amount of impurities in brain extracts were such that a single purification step was inadequate and a combination of CM- and DEAE-cellulose chromatographies was needed before defined focusing patterns could be obtained.

Band 0 seen in fibroblasts has been enigmatic. Its presence in Maroteaux-Lamy fibroblasts (deficient in arylsulfatase B [17]) and its staining with the arylsulfatase A specific assay reagent of Baum et al. [5] indicated the presence of an A-type arylsulfatase. Band 0 was also present (located by the fluorogenic substrate) in metachromatic leukodystrophy fibroblasts which are essentially deficient in arylsulfatase A [18]. In these fibroblasts, Band 0 does not stain with the arylsulfatase A specific reagent. We have concluded, therefore, that Band 0 is composed of a mixture of an unique species of arylsulfatase A and a form of anionic arylsulfatase B-like enzyme [6]. There have been suggestions of material in the region of Band 0 in extracts from some tissues, but they were very faint and could only be seen sporadically, even under condi-

tions of heavy loading. The unique occurrence of significant amounts of Band 0 activity in fibroblast extracts is interesting because this is the only fresh, actively growing tissue examined. Presently developing concepts concerning the biosynthesis of lysosomal enzymes, such as arylsulfatase A, include a "maturation" phase after assembly of the basic polypeptide chain. Thus, it is conceivable that a prelysosomal form of the enzyme is present in increased amounts in actively growing tissue and its occurrence in urine or postmortem tissues is minimal or absent. Whether the Band 0 enzyme of fibroblasts represents such a transitional form of arylsulfatase A is conjectural, but information concerning such species will be necessary in understanding certain sulfatase deficiency diseases.

The apparent uniform stepwise change in p*I* values of enzyme in Bands 1—6 could occur by an integral charge difference between successive components. Amino acid analysis of the urinary arylsulfatase A has shown 88 basic and 160 acidic amino acid residues per molecular weight of 100 000 (unpublished observations). Since the enzyme has an overall p*I* of 4.8, we have estimated that about 60 of the acidic groups exist as amides. Calculations based on this premise show that a unit charge change would shift the p*I* about 0.05 units *. By independent experimental methods an average shift in p*I* of 0.06—0.1 per unit charge change was observed by ^{14}C-carbamoylation of amino groups of arylsulfatase A (unpublished data). The observed p*I* spacing of the arylsulfatase A bands are therefore consistent with differences of one or two charges per molecule. Such charge changes have been noted to result from selective deamidation of glutamine or asparagine residues, esterification by phosphate or sulfate groups, or addition of organic acids such as neuraminic acid. The shift of the six-banded pattern of liver enzyme to the three-banded pattern of urine enzyme by neuraminidase treatment implies that neuraminic acid residues are involved in some but not all of the charge microheterogeneity of arylsulfatase A.

Arylsulfatase A from urine appears to be composed of two subunits of identical size [1]. If the subunits were chemically non-identical and all combinations of two subunits were enzymatically competent, the three-banded pattern on isoelectric focusing would be expected. The absence of all bands in the recessively transmitted disorder metachromatic leukodystrophy implies a single gene locus for arylsulfatase A. Consequently, these forms of arylsulfatase A are not analogous to the classical isoenzymes with separate loci for each subunit and combinations of subunits to accommodate differing metabolic requirements. The most likely explanation of the microheterogeneity of arylsulfatase A is a series of post-synthetic modifications of the putative gene product which includes sialylation. These modifications may represent a mechanism to facilitate tissue specific compartmentalization.

---

* The estimate of 60 amide groups was based on an assumed enzyme carboxyl p*K* of 4.0. This value is the average p*K* of the carboxyl groups of albumin [19] which has a p*I* similar to that of arylsulfatase A. If the assumed amide content is increased or decreased within reasonable limits (±10%) the change in p*I* per unit charge only varies between 0.03 and 0.1 pH units.

Acknowledgements

We are indebted to Drs. R.K. Draper and J. Edmond for kindly providing us with a sample of purified human liver arylsulfatase A and to the Department of Chemical Research, Hoffman-La Roche Inc. for a generous supply of ascorbic acid 2-sulfate. This investigation was supported in part by NIH grants NS-11665, NS-9479 and HD-4612.

References

1 Stevens, R.L., Fluharty, A.L., Skokut, M.H. and Kihara, H. (1975) J. Biol. Chem. 250, 2495—2501
2 Fluharty, A.L., Davis, M.L., Kihara, H. and Kritchevsky, G. (1974) Lipids 9, 865—869
3 Rinderknecht, H., Geokas, M.C., Carmack, C. and Haverback, B.J. (1970) Clin. Chim. Acta 29, 481—491
4 Finlayson, G.R. and Chrambach, A. (1971) Anal. Biochem. 40, 292—311
5 Baum, H., Dodgson, K.S. and Spencer, B. (1959) Clin. Chim. Acta 4, 453—455
6 Stevens, R.L. (1974) Biochim. Biophys. Acta 370, 249—256
7 Draper, R.K. and Edmond, J. (1973) Fed. Proc. 32, 553
8 Porter, M.T., Fluharty, A.L., de la Flor, S.D. and Kihara, H. (1972) Biochim. Biophys. Acta 258, 769—778
9 Fluharty, A.L., Stevens, R.L., Miller, R.T., Shapiro, S.S. and Kihara, H. (1976) Biochim. Biophys. Acta 429, 508—516
10 Price, R.G. and Dance, N. (1972) Biochim. Biophys. Acta 271, 145—153
11 Thorpe, R. and Robinson, D. (1975) FEBS Lett. 54, 89—92
12 Alhadeff, J.A., Miller, A.L., Wenaas, H., Vedvick, T. and O'Brien, J.S. (1975) J. Biol. Chem. 250, 7106—7113
13 Stevens, R.L., Hartman, M., Fluharty, A.L. and Kihara, H. (1973) Biochim. Biophys. Acta 302, 338—344
14 Neuwelt, E., Stumpf, D., Austin, J. and Kohler, P. (1971) Biochim. Biophys. Acta 236, 333—346
15 Harzer, K., Stinshoff, K., Mraz, W. and Jatzkewitz, H. (1973) J. Neurochem. 20, 279—287
16 Eto, Y., Wiesmann, U. and Herschkowitz, N.N. (1974) J. Biol. Chem. 249, 4955—4960
17 Fluharty, A.L., Stevens, R.L., Sanders, D.L. and Kihara, H. (1974) Biochem. Biophys. Res. Commun. 59, 455—461
18 Porter, M.T., Fluharty, A.L. and Kihara, H. (1969) Proc. Natl. Acad. Sci. U.S. 62, 887—891
19 Tanford, C. (1961) in Physical Chemistry of Macromolecules, p. 560, John Wiley and Sons, New York