Purification and Properties of Arylsulfatase A from Human Urine

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SUMMARY

Arylsulfatase A (cerebroside sulfate sulfohydrolase) was purified 3500-fold at a 7% yield from human urine. A crude urinary protein concentrate was prepared by treating pooled urine with ammonium sulfate and subsequently drying the precipitate with acetone. The powder thus obtained was extracted with buffer and was subjected to chromatographic and electrophoretic procedures as follows: (a) ammonium sulfate reverse gradient solubilization chromatography; (b) DEAE-cellulose chromatography; (c) Sephadex G-200 gel filtration; (d) preparative polyacrylamide gel electrophoresis; (e) Sephadex chromatography; and (f) antialbumin-Sepharose chromatography. The enzyme was judged to be essentially homogeneous by: (a) a single band on polyacrylamide gel electrophoresis at two pH values; (b) formation of a single precipitin line on immunodiffusion against its antiserum; (c) complete freedom from albumin, the major contaminating protein; and (d) a single band on sodium dodecyl sulfate gel electrophoresis.

The molecular weight of the native enzyme is near 100,000, whereas the reduced, denatured enzyme has a molecular weight around 50,000. Thus arylsulfatase A appears to be composed of two subunits of identical size. The kinetic constants \( K_m, V_{max}, pH \) optimum, and the energy of activation were determined for the substrates, 4-nitrocatechol sulfate, 4-methylumbelliferyl sulfate, and cerebroside sulfate. With the synthetic substrate, 4-nitrocatechol sulfate, the urine enzyme shows the same anomalous kinetics exhibited by the enzyme from other sources. Activity toward the physiological substrate, cerebroside sulfate, was elicited only in the presence of either sodium taurodeoxycholate or sodium cholate with no obligatory requirement for other factors.

EXPERIMENTAL PROCEDURE

Materials--4-Methylumbelliferyl sulfate was synthesized by the procedure of Sherman and Stanfield (20) and was purified according to Rinderknecht et al. (21). [\( ^{14}C \)]Cerebroside sulfate was biosynthesized and isolated from rat brain (22). Sephadex G-200, Sephadex C-50, and Sepharose 6B were obtained from Pharmacia Fine Chemicals, Inc.; DEAE-cellulose as Cellex-D and polyacrylamide gel reagents from Bio-Rad Laboratories; Coomassie brilliant blue R 250 from Colab Laboratories, Inc.; Celite analytical grade filter aid from Johns-Manville Products Corp.; rabbit antiserum against human serum albumin from Hyland; molecular weight markers, cytochrome c, myoglobin, ovalbumin, crystalline albumin, \( \gamma \)-globulin, and apotitorin from Schwarz-Mann; bovine albumin Fraction V from Pierce Chemical Co.; 4-nitrocatechol sulfate, sodium dodecyl sulfate (lauryl sulfate, sodium salt), Tris, and ribonuclease A from Sigma Chemical Co.; and dithiothreitol, TES, triethanolamine, glyceraldehyde-3-phosphate dehydrogenase, and insulin (recrystallized) from Calbio-

Cerebroside sulfatase (cerebroside-3-sulfate 3-sulfohydrolase, EC 3.1.6.8, commonly known as arylsulfatase A or sulfatase A) is deficient in the genetic sphingolipidosis, metachromatic leukodystrophy (1-3). The enzyme deficiency results in the accumulation of cerebroside sulfate in the tissues of affected patients, producing severe neurological consequences (4, 5). There are several clinical forms of the disorder, distinguishable by the latency of the manifestation of clinical symptoms (6). A variety of distinct aberrations in the cerebroside sulfatase molecule is implied by these different forms of the disorder (7). Characterization of such modified enzymes is dependent on a detailed understanding of the normal enzyme.

Arylsulfatase A has been isolated in pure form from beef liver (8), beef brain (9), pig kidney (10), and rabbit liver (11), and parameters such as physicochemical properties and catalysis of hydrolysis of synthetic substrates have been investigated extensively. There is a paucity of comparable studies with pure human enzyme, but a number of partially purified enzyme preparations from human source, including brain (12, 13), liver (14), kidney (15), and urine (16), have been described. We are aware of only two preparations, one from placenta (17) and the other from liver (18, 19), in which specific activities approximate those of pure animal preparations. Urine, although not rich in enzyme, was chosen as the source material for the purification of human arylsulfatase A because of its readily availability.

The urinary enzyme was isolated and purified to a homogenous form as determined by several criteria. The kinetic data of the purified enzyme were explored with the substrates 4-nitrocatechol sulfate, 4-methylumbelliferyl sulfate, and cerebroside sulfate. Other properties of arylsulfatase A such as molecular weight, subunit formation, and reaction with antibody also were investigated.

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The urinary enzyme was isolated and purified to a homogenous form as determined by several criteria. The kinetic data of the purified enzyme were explored with the substrates 4-nitrocatechol sulfate, 4-methylumbelliferyl sulfate, and cerebroside sulfate. Other properties of arylsulfatase A such as molecular weight, subunit formation, and reaction with antibody also were investigated.

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1 The abbreviation used is: TES, N-tris(hydroxymethyl)methyl-

2-aminethanesulfonic acid.

* This study was supported in part by Grants NS-9439, NS-8839, HD-4612, and RR-5632 from the National Institutes of Health. United States Public Health Service.
chern. Bulk ammonium sulfate "certified A.C.S." was obtained from Fisher Scientific Co. Ultrafiltration membranes were either the XM-50 type from Amicon Corp. or the collodion bags as supplied by Schleicher and Scuell, Inc.

Assay for Enzymatic Activity—Assays for enzyme activity during the purification were performed with a modified (23) arylsulfatase A specific procedure of Baum et al. (24) using 4-nitrocatechol sulfate as the substrate with a 30-min incubation. Activity toward 4-methylumbelliferyl sulfate was determined by a procedure similar to that of Harinath and Robins (25). The reaction mixture, containing 5 mm substrate in 50 mm sodium acetate, pH 5.5, with 50 mm NaCl and enzyme in a total volume of 100 μl, was incubated for 1 hour at 37°C. The reaction was stopped with 1.0 ml of 400 mm glycine-NaOH, pH 10.5, and the fluorescent determination at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. Activity toward cerebroside sulfate was determined by an estimation of the amount of [18]sulfate released (26). A unit of enzyme activity is defined as the amount of enzyme which catalyzes the hydrolysis of 1 μmol of substrate/hour under the present assay conditions. Protein estimations of chromatographic fractions were derived from absorption at 280 nm (27) or from fluorescence at an excitation wavelength of 290 nm and an emission wavelength of 450 nm (28). Specific activity of the enzyme at each fractionation step was based on the 4-nitrocatechol sulfate assay and protein determination on pooled fractions by the method of Lowry et al. (29). Bovine serum albumin was used as the standard for protein determinations.

Analytical Polyacrylamide Gel Electrophoresis—The procedure used to examine enzyme preparations by gel electrophoresis was the trichloroacetic-TES gel system at pH 6.8 as described by Orr et al. (30). Enzyme bands were located by placing gels in 4-methylumbelliferyl sulfate (10 mm in 500 mm sodium acetate, pH 5.3) and visualizing the fluorescent product, 4-methylumbelliferone, under a long wavelength ultraviolet lamp (365 nm maximum). The enzyme band and the bromphenol blue front were marked by stabbing the gels with India ink; then the same gels were stained for protein with Coomassie blue in 12.5% trichloroacetic acid.

Preparative Polyacrylamide Gel Electrophoresis—The Canalo preparative disc electrophoresis apparatus was used with the trichloroacetic-TES gel system of Orr et al. (30). The gel formation was modified by increasing the ammonium persulfate to 2 mg/ml and by decreasing prior to casting at 4°C. The upper (cathode) buffer (pH 6.3), resolving gel buffer (pH 6.8), and lower (anode) buffer (pH 5.8) system used also were as described by Orr et al. (30), but EDTA was omitted and 1 mm thiglycolic acid was added to the upper buffer. The chution buffer was 150 mm trichloroacetic-HCl, pH 7.0. The apparatus was placed in a 4°C cold room and coolant at 4°C was circulated in the jacket. The electrophoresis was carried out at 130°C and 300 volts.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—Protein solutions were denatured by (a) 1% sodium dodecyl sulfate with 1% β-mercaptoethanol in 10 mM phosphate buffer, pH 7.0, for 2 hours at 37°C or (b) the same protocol with 5 mm dithiothreitol replacing the β-mercaptoethanol. The mixtures were subjected to electrophoresis in polyacrylamide gels according to Weber and Osborn (31).

Preparation of Antiserum—Complete Freund's adjuvant and 1 μg of purified arylsulfatase A in 0.1 ml were injected through the skin into the popliteal lymph node of one leg of long-eared male albino New Zealand rabbits. Booster injections were administered at approximately 2-week intervals with the same amount of enzyme in 1 ml of the adjuvant divided into several intramuscular and subcutaneous injections.

Preparation of Antialbumin-Sepharose—Rabbit antiserum against human serum albumin was coupled to cyanogen bromide-activated Sepharose 6B as described by Cantz et al. (32). The capacity of the conjugated Sepharose equilibrated with 100 mm Tris-HCl, pH 7.5, was 85 μg of albumin/ml of packed Sepharose. Ultrafiltration—Ultrafiltration was kindly performed by Dr. Gary Hathaway in a Spinco model G analytical ultracentrifuge. High speed sedimentation equilibrium centrifugation using interference optics was conducted essentially as described by Yphantis (33). The system was brought to equilibrium with protein at an initial concentration of 0.8 mg/ml in 10 mm Tris-HCl, pH 7.5, containing 150 mm NaCl at 15°C at 15,220 rpm. Attainment of equilibrium was ascertained experimentally. Molecular weight values for maximum and minimum size components were calculated by extrapolation of curves obtained from experimental points at the bottom and top of the cell.

RESULTS

Purification of Arylsulfatase A

Step 1: Concentration of Urinary Proteins and Preparation and Extraction of Acetone Powder—Urine collected from laboratory personnel over 24-hour periods was pooled each morning and solid (NH₄)₂SO₄ added with stirring to 2.8 M. After settling for 24 hours at 4°C, the bulk of the supernatant solution was removed by aspiration and the remainder was centrifuged at 500 × g for 30 minutes. Four volumes of cold (4°C) acetone were added to the precipitated material and stirred for 30 minutes. The mixture was filtered with the aid of a aspirator and the material was washed successively in the Buchner funnel with 2 volumes of acetone, 2 volumes of acetone-diethyl ether (1:1, v/v), and 2 volumes of diethyl ether. The acetone powder thus obtained was air dried and stored at −20°C. Acetone powder, 200 g, representing 80 liters of urine, was extracted successively with 400-ml portions of 20 mm Tris-HCl, pH 7.5 (Buffer A), by stirring for 30 minutes and centrifuging at 10,000 × g for 10 minutes. The bulk of the soluble protein as well as the enzyme usually was present in the second extract. Recovery of the protein and enzyme in this and subsequent steps is summarized in Table I.

Step 2: Ammonium Sulfate Reverse Gradient Solubilization Chromatography—The second extract of the acetone powder was diluted to 800 ml with Buffer A to adjust the protein concentration to 1 to 2 mg/ml. Colite, 150 g, was added; then 450 g of (NH₄)₂SO₄ were added with stirring. The mixture was left overnight at 4°C and was poured into a 5-cm diameter column forming a packed height of approximately 26 cm. From this step all operations were carried out at 4°C. The column was eluted with a decreasing linear ammonium sulfate gradient formed with 1140 ml of 80% saturated (3.28 M) (NH₄)₂SO₄ in Buffer A in the mixing chamber and 1320 ml of buffer in the reservoir chamber. The flow rate was maintained at 120 ml/hour and 10-ml fractions were collected. As seen in Fig. 1, over 90% of the enzyme was recovered between 25 and 40% saturated (1.00 and 1.60 M)

### Table I

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Recovery</th>
</tr>
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<tbody>
<tr>
<td>Urine, 80 liters</td>
<td>(8000)⁺</td>
<td>1270</td>
<td>6.5</td>
<td>100</td>
</tr>
<tr>
<td>1. Acetone powder extract</td>
<td>4740</td>
<td>1436</td>
<td>3.3</td>
<td>60</td>
</tr>
<tr>
<td>2. (NH₄)₂SO₄ Reverse gradient</td>
<td>3080</td>
<td>306</td>
<td>10</td>
<td>38</td>
</tr>
<tr>
<td>3. DEAE-cellulose</td>
<td>2705</td>
<td>67</td>
<td>45</td>
<td>34</td>
</tr>
<tr>
<td>4. Sephadex G-200</td>
<td>1552</td>
<td>13</td>
<td>116</td>
<td>10</td>
</tr>
<tr>
<td>Pooled Step 4</td>
<td>3520</td>
<td>35</td>
<td>100</td>
<td>19</td>
</tr>
<tr>
<td>5. Preparative polyacrylamide electrophoresis</td>
<td>2008</td>
<td>2.29</td>
<td>878</td>
<td>11</td>
</tr>
<tr>
<td>6. SP-Sephadex</td>
<td>1140</td>
<td>0.40</td>
<td>3000</td>
<td>8</td>
</tr>
<tr>
<td>7. Antialbumin-Sepharose</td>
<td>1270</td>
<td>0.36</td>
<td>3520</td>
<td>7</td>
</tr>
</tbody>
</table>

⁺Enzyme activity was not actually measured on the pooled urine sample as it was collected and processed over a period of several weeks. In an extensive series of measurements an average value of 100 units of arylsulfatase A per liter at a specific activity of 1 unit/mg of protein was observed, and these averages were used to estimate enzyme in the original urine.
(NH₄)₂SO₄ as arylsulfatase A in the α form (34). Less than 5% of the total enzyme was recovered in the β form between 45 and 60% saturated (1.8 and 2.5 M) (NH₄)₂SO₄. The fractions indicated "pool" in Fig. 1, representing approximately 95% of the enzyme, were combined and dialyzed against Buffer A.

Step 3: DEAE-Cellulose Chromatography—The dialyzed enzyme solution (765 ml) was applied to a DEAE-cellulose column (5 × 26 cm) which had been equilibrated with Buffer A. Arylsulfatase A was eluted at 120 ml/hour with 2 liters of a linear gradient of NaCl ranging from 0 to 600 mM in Buffer A and was collected in 12-ml fractions as shown in Fig. 2. Fractions containing 90% of the enzyme were pooled and concentrated to 20 ml by ultrafiltration on a Diaflo XM-50 membrane in an Amicon 202 ultrafiltration cell.

Step 4: Sephadex G-200 Gel Filtration—The enzyme concentrate was subjected to gel filtration by reverse flow at 20 ml/hour on a Sephadex G-200 column (2.5 × 93 cm) which had been equilibrated with 10 mM Tris-HCl, pH 7.0, containing 50 mM NaCl, and 5-ml fractions were collected (Fig. 3). Fractions containing approximately 95% of the enzyme were pooled and concentrated by ultrafiltration to about 3 ml.

Step 5: Sephadex G-200 Gel Filtration—The enzyme concentrate from the gel filtration step from several preparations was pooled for electrophoresis. To a 2-ml sample containing 3520 units of arylsulfatase A with a specific activity of 100, 200 mg of sucrose and 5 µl of 0.5% bromphenol blue were added. This mixture was layered under the top buffer onto a 4-cm column of separating gel. Elution was carried out at 30 ml/hour and 2.5-ml fractions were collected (Fig. 4). Fractions containing approximately 88% of the enzyme were pooled and concentrated by ultrafiltration to about 3 ml.

Step 6: SP-Sephadex Chromatography—The enzyme concentrate from the electrophoresis step was adsorbed onto a SP-Sephadex column (1.5 × 12 cm) which had been equilibrated with 10 mM sodium acetate, pH 5.0, containing 20 mM NaCl. The enzyme was desorbed with buffer containing 50 mM NaCl at 15 ml/hour and 1-ml fractions were collected (Fig. 5). The bulk of the protein emerged with the void volume. The enzyme-containing fractions were pooled and concentrated by ultrafiltration to 1.7 ml.

Step 7: Removal of Albumin by Affinity Chromatography on Antialbumin-Sepharose—Immunochemical examination had indicated that the principal contaminating protein even after the gel filtration step was albumin. The preparative electrophoresis and SP-Sephadex chromatography steps had removed a large amount of this contaminant, but the preparation still contained albumin. Antialbumin-Sepharose with a binding capacity for 500 µg of human albumin was packed into a column (0.8 × 12
to 135,000 range (cf. Fig. 3). By sedimentation equilibrium ultra centrifugation, the molecular weight of the material at the bottom of the cell was estimated at approximately 100,000, utilizing a partial specific volume of 0.715 as determined by Nichol and Roy (38) for the bovine enzyme. The ultracentrifuge data also suggested that about 30% of the material was present as a species of approximately 50,000 molecular weight. The enzyme recovered after the centrifugation showed 72% of the activity of the initial enzyme solution.

The enzyme migrated as a single sharp band on polycrylamide gel electrophoresis in the presence of sodium dodecyl sulfate with an apparent molecular weight of 55,000. Fig. 7 shows that only one band of protein was present after the enzyme was treated with 1% sodium dodecyl sulfate-1% β-mercaptoethanol. Identical results on gel electrophoresis also were obtained when the enzyme was subjected to 1% sodium dodecyl sulfate-5 mM dithiothreitol. Thus, arylsulfatase A appears to contain two subunits of the same or nearly the same size.

**Stability**—Arylsulfatase A in urine or in crude preparations is remarkably stable even at relatively low protein concentrations. As the enzyme is purified, dilute solutions (<0.1 mg of protein/ml) are quite unstable. Very dilute enzyme solutions (2 µg/ml) with adducts such as ribonuclease, bovine serum albumin, or insulin (1 mg/ml in 25 mM Tris-HCl, pH 7.5) retained complete activity after 15 days at 4° and greater than 70% of the activity after 8 months. On the other hand, with adducts such as dithiothreitol, glutathione, or EDTA (1 mM) approximately 1/3 of the activity was lost after 15 days at 4° and no activity remained after 8 months. Enzyme solutions at protein concentrations in the range of 0.5 to 1.0 mg/ml or dilute enzyme solutions with a protein adduct such as bovine serum albumin (1 mg/ml) were stable for months at -20° at neutral pH.

**Kinetic Parameters**—The pH optimum, K, and Vmax toward two synthetic substrates, 4-nitrocatechol sulfate and 4-methylumbelliferyl sulfate, and the physiological substrate, cerebroside sulfate, are shown in Table II. Assuming a molecular weight of 100,000 for arylsulfatase A, the apparent zero order rate constants are 1010, 49, and 87 min⁻¹ for 4-nitrocatechol sulfate, 4-methylumbelliferyl sulfate, and cerebroside sulfate, respectively. The ratio of activity toward the three substrates was essentially unchanged throughout the purification.

The anomalous kinetic data in the hydrolysis of 4-nitrocatechol sulfate are characteristic of arylsulfatase A from many sources, and the present purified enzyme shares these characteristics. The time course of the reaction under the present standard assay condition of Baum et al. (24) does not follow zero order kinetics (Fig. 8). Instead, a biphasic reaction rate, an initial fast rate, and a later manifesting slower rate appear to be occurring (Curves A and B). A comparison of the reactions at two levels of enzyme shows near but not precise proportionality with enzyme concentration. Examination of the time course of the reaction under the assay conditions of Roy (40), which differ from those of Baum et al. (24) by the omission of pyrophosphate and NaCl, showed that the initial rate was faster, but after 15 min the reaction essentially terminated, presumably due to enzyme inactivation (Curves C and D). The latter reaction for 1 min frequently is used for the estimation of specific activity and in the present instance this yields a value of 10,300 mits/mg of protein.

The rate of hydrolysis of 4-methylumbelliferyl sulfate was linear with respect to arylsulfatase A concentration between 1 and 100 µg. The reaction also was linear up to 30 min, and at 60 min the rate of hydrolysis was 85% of the extrapolated linear rate.
A, comparison of protein bands of a partially purified enzyme solution in two different pH systems. Each gel was charged with 12 μg of the same enzyme solution of 218 units/mg of protein. The enzyme activity band, denoted E, and the bromphenol blue front, denoted F, were marked with India ink. The gels subsequently were stained with Coomassie blue and were photographed after overnight destaining. Gel 1, pH 8.9, Tris-glycine buffer system; Gel 2, pH 6.8, TES-triethanolamine buffer system. B, enzyme stain of arylsulfatase A samples at various stages of purity in the pH 6.8 TES-triethanolamine buffer system. The gels were photographed under ultraviolet light after a few minutes of incubation at room temperature with 4-methylumbelliferyl sulfate at pH 5.3.

**TABLE II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
<th>pH Optima</th>
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<tbody>
<tr>
<td>4-Nitrocatechol sulfate</td>
<td>6.7</td>
<td>5950</td>
<td>5.3</td>
</tr>
<tr>
<td>4-Methylumbelliferyl sulfate</td>
<td>8.0</td>
<td>310</td>
<td>5.2</td>
</tr>
<tr>
<td>Cerebroside sulfate</td>
<td>0.2</td>
<td>550</td>
<td>4.6</td>
</tr>
</tbody>
</table>

* The Michaelis-Menten parameters, $K_m$ and $V_{max}$, were calculated from Lineweaver-Burk reciprocal plots (39).

Inasmuch as some enzymes acting on hydrophobic substrates show unusual responses to changes in reaction temperature, the effect of temperature on arylsulfatase A activity toward the two synthetic substrates and the physiological substrate was examined (Table III). The maximum rate of hydrolysis of all substrates was decreased at 46°, and the rate for 4-nitrocatechol sulfate at 37° also appeared to be somewhat depressed. Estimations of the energy of activation from Arrhenius plots of $V_{max}$ at the lower temperatures gave values of 12.4, 8.6, and 21.5 Cal/mol for 4-nitrocatechol sulfate, 4-methylumbelliferyl sulfate, and cerebroside sulfate, respectively. With the two synthetic substrates, the $K_m$ also varied with temperature. The $K_m$ decreased when the temperature was increased with an overall 5- to 6-fold change over the temperature range from 18-46°. In contrast, the $K_m$ for cerebroside sulfate did not change with a change in temperature.

**DISCUSSION**

**Purification**—Arylsulfatase A was isolated from human urine with a 3500-fold increase in specific activity at an overall yield
ence of arylsulfatase A in urine in two forms, A, and AD, we found enzyme purification applications. In the study showing the pres-

Arylsulfatase A hydrolysis of different substrates with respect to temperature

<table>
<thead>
<tr>
<th>Temperature</th>
<th>4-Nitrocatechol sulfate</th>
<th>4-Methylumbelliferyl sulfate</th>
<th>Cerubroside sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{max}^a$ units/mg</td>
<td>$V_{max}^b$ units/mg</td>
<td>$V_{max}^c$ units/mg</td>
</tr>
<tr>
<td>18</td>
<td>2940</td>
<td>167</td>
<td>167</td>
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<tr>
<td>24</td>
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<tr>
<td>46</td>
<td>2960</td>
<td>303</td>
<td>303</td>
</tr>
</tbody>
</table>

* The maximum velocities were calculated from Lineweaver-Burk reciprocal plots (39).

* The highest activity recorded in the temperature series was arbitrarily assigned a value of 100%.

of 7%. The final arylsulfatase A preparation was judged to be essentially homogeneous by the following criteria: (a) a single band on polyacrylamide gel electrophoresis at pH 8.8 and 8.8 with coincidence of protein and enzyme activity; (b) formation of a single precipitin line on immunodiffusion against its anti-

ammonium sulfate reverse gradient solubilization tech-
nique permitted recovery of the enzyme in a narrower ammonium sulfate concentration range than the traditional stepwise precipita-
tion technique. Carry-over of contaminant proteins was decreased and higher specific activities were achieved. These find-
ings are in accord with King (41), who described the technique with isolated proteins and predicted its effectiveness in actual enzyme purification applications. In the study showing the pres-

ence of arylsulfatase A in urine in two forms, $A_a$ and $A_b$, we found that this technique was equally effective on an analytical scale (34).

The major proportion of the protein in urine is albumin. Album-
in, like arylsulfatase A, is anionic and in the dimeric form its molecular weight is nearly equivalent to that of arylsulfatase A. As a consequence, the behavior of albumin in purification procedures based on size or charge did not differ greatly from that of arylsulfatase A. The elution profiles of the chromatographic procedures (cf. Figs. 1 to 3) show that the major protein peaks were displaced from the enzyme peaks, but because of the bulk of the protein there was considerable overlap into the enzyme-containing fractions. The preparative polyacrylamide gel electrophoresis step was quite effective for bulk albumin removal (cf. Fig. 4), but there was still carryover. Repeating the electrophoresis step did serve to decrease the amount of albumin in the en-

zyme fractions. However, in spite of various modifications, en-
zyme recovery through this procedure was poor.

Inasmuch as human arylsulfatase A is an anionic protein, it would not be expected to bind cation exchange resins at pH levels above its pI of 4.7 (15, 42). However, the sulfo group of SP-Sepha-
dex, a sulfopropyl cation exchanger, suggested a potential for affinity type binding to sulfate receptor sites of the enzyme. In-
vestigations oriented to this possibility indicated that there was indeed an affinity of the enzyme for SP-Sephadex at pH 5.0 (43). Although most of the albumin was separated from the en-
zyme by SP-Sephadex treatment, its removal still was not com-
plete. The tenacious contamination of certain urinary enzymes by albumin had been noted by Cantz and others (32). They had used antialbumin-Sepharose affinity chromatography as an effective procedure to remove albumin from their sulfodiuronate sulfatase preparation. Its application in the final step removed all traces of albumin from the arylsulfatase A as evidenced by immuno-

chemical evaluation.

The molecular weight of arylsulfatase A was estimated to be approximately 130,000 by gel filtration, whereas a value of 100,000 was obtained by sedimentation equilibrium centrifuga-
tion. The human urinary enzyme, like the beef liver enzyme (44), is a glycoprotein as judged by its affinity to concanavalin A-Sepharose, so the gel filtration value probably would be an overestimation. The lower value is similar to the molecular weight of 107,000 reported for the monomeric beef liver enzyme (38) derived by the Archibald and equilibrium ultracentrifugation methods and the 104,500 value derived by sedimentation equilib-
rium for the monomeric human liver enzyme (45).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis with either mercaptoethanol or dithiothreitol as the reducing agent gave subunits of approximately 55,000 molecular weight. Similar size subunits have been found for the beef liver enzyme by Roy and Jerfy (48). Nichol and Roy (47) have suggested that there may be a further breakdown to 25,000 molecular weight units, but the urinary enzyme has given no indication of subunits smaller than one-half of the monomeric enzyme.

In the ultracentrifugation study about 30% of the total material behaved as a component of approximately 55,000 molecular weight; furthermore, only 70% of the added enzyme activity could be recovered from the ultracentrifugation cell. Gel electrophoresis of the enzyme before and after centrifugation yielded only a single protein band. It thus appears that the loss of 30% of the enzyme on ultracentrifugation, the presence of 30% of the material as a species of approximately 50,000 molecular weight, and the 55,000 molecular weight as the subunit size are

* R. L. Stevens, unpublished observations.
more than a fortuitous coincidence. It is believed that during centrifugation there was local dilution and dissociation of the enzyme accompanied by an irreversible loss of activity. A more detailed re-evaluation is required but can only be pursued as purified enzyme in larger quantities is prepared.

The catalytic properties of the human urinary arylsulfatase A were indistinguishable from those of animal enzyme. The same anomalous kinetic data were exhibited with 4-nitrocatechol sulfate and the requirements and conditions for the cerebroside sulfate hydrolase reaction were identical with those for the beef liver enzyme (48). With either enzyme, hydrolysis of the sulfoplipid occurred in the presence of a bile salt without any supplemental protein component such as was suggested by the complementary factor of Mehl and Jatzkewitz (3). Jatzkewitz and Stinshoff (49) have more recently purified and redesignated the factor as an “activator” which apparently enhances the sulfohydrolase reaction beyond that by bile salts.

Values for the energy of activation of 12.4 and 8.6 Cal/mol found for 4-nitrocatechol sulfate and 4-methylumbelliferyl sulfate are similar to the values of 6 to 14 Cal/mol observed with beef liver enzyme under various conditions (50). With a given enzyme the same activation energy often is obtained for all substrates. Arylsulfatase A does not appear to follow this pattern, because the energy of activation for cerebroside sulfate was found to be 21.5 Cal/mol, which is about twice that for the synthetic hydrolase reaction beyond that by bile salts. The catalytic properties of the human urinary arylsulfatase A and the requirements and conditions for the cerebroside sulfate appearance to be quite complex, and the significance of the elevated activation energy cannot be rationalized at this time.

A protocol for the preparation of pure human urinary arylsulfatase A has been developed. A systematic investigation of the properties of normal enzyme can be pursued to provide guidelines for the characterization of mutant forms of arylsulfatase A produced in metachromatic leukodystrophy.

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