Purification and Properties of Arylsulphatase A from Rabbit Testis

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Rabbit testis arylsulphatase A was purified 140-fold with a recovery of 20% from detergent extracts of an acetone-dried powder by using DE-52 cellulose column chromatography, gel filtration on Sephadex G-200 and preparative isoelectric focusing. The purified enzyme showed one major band with one minor contaminant on electrophoresis in a 7.5% (w/v) polyacrylamide gel at pH 8.3. On sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, a single major band was observed with minor contaminants. The final preparation of enzyme was free from general proteolytic, esterase, hyaluronidase, β -glucuronidase and β -galactosidase activities. Rabbit testicular arylsulphatase A exists as a dimer of mol.wt. 110000 at pH 7.1. At pH 5.0 the enzyme is a tetramer of mol.wt. 220000. Arylsulphatase A appears to consist of two identical subunits of mol.wt. 55000 each. The highly purified enzyme has pI 4.6. The enzyme hydrolyses *p*-nitrocatechol sulphate with K_m and V_{max} of 4.1 mM and 80 nmol/min respectively, but has no activity toward *p*-nitrophenyl sulphate. The pH optimum of the enzyme varies with the incubation time. By applying Sephadex G-200 chromatography and preparative isoelectric focusing, one form of enzyme was obtained. The enzyme has properties common to arylsulphatase A of other sources with respect to the anomalous time-activity relationship, pI, inhibition by PO_4^{2-} , SO_3^{2-} , SO_4^{2-} and Ag^+ ions and substrate affinity to *p*-nitrocatechol sulphate. However, the enzyme shows the temperature optimum of arylsulphatase B of other species.

The arylsulphatase enzymes (arylsulphate sulphonydrolase, EC 3.1.6.1) were identified in mammalian testis (Dodgson *et al.*, 1953; Hook *et al.*, 1973) and in spermatozoa acrosomes (Allison & Hartree, 1970). Those enzymes were partially purified from spermatozoa acrosomes (Yang & Srivastava, 1974a) and from testis (Yamato *et al.*, 1974; Yang & Srivastava, 1975a). Several mammalian tissues contain at least two or three distinct enzymes (arylsulphatases A, B and C) capable of hydrolysing arylsulphate ester linkages (Dodgson *et al.*, 1956). Arylsulphatases A and B can be distinguished by their different pH optima, substrate affinities, anomalous kinetics and electrophoretic mobilities (Farooqui & Bachhawat, 1972). Arylsulphatase isoenzymes from bacterial sources can be characterized by their electrophoretic mobilities (Delisle & Milazzo, 1972). Arylsulphatases A and B can be separated by fractional precipitation with acetone (Roy, 1953), by paper electrophoresis (Roy, 1958) and by gel filtration on Sephadex G-200 (Wynn, 1966; Worwood *et al.*, 1973). The independent assay for arylsulphatases A and B is established for enzymes from human organs (Baum *et al.*, 1958) and ox brain (Dzialoszynski *et al.*, 1966). Worwood *et al.* (1973) suggested that arylsulphatases A and B should be separated before determination of the individual activities. A natural substrate such as cerebroside 3-sulphate is hydrolysed by arylsulphat-

ase A from pig kidney and ox liver (Mehl & Jatzkewitz, 1968), chicken brain, human brain (Farooqui & Bachhawat, 1972) and human urine (Stevens *et al.*, 1975). Yamato *et al.* (1974) demonstrated that arylsulphatase A from boar testis hydrolysed a sulphated glyceroglycolipid obtained from boar testis and spermatozoa. The present paper describes (1) the methods for obtaining highly purified testicular arylsulphatase, (2) the characterization of the enzyme and (3) the evidence that testicular arylsulphatase exists in only one form as shown by isoelectric-focusing data but that the enzyme has properties common to both arylsulphatases A and B.

Materials and Methods

Materials

Rabbit testes were purchased from Pel-Freez Biologicals, Rogers, AR, U.S.A. The dipotassium salt of 2-hydroxy-5-nitrophenyl sulphate, potassium *p*-nitrophenyl sulphate, *p*-nitrophenyl β -D-galactoside, phenolphthalein mono- β -glucuronide (sodium salt) and α -N-benzoyl-L-arginine ethyl ester hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Azocoll, a general proteolytic substrate, and pepsin as standard protein for sodium dodecyl sulphate/polyacrylamide-gel electro-

phoresis were purchased from Calbiochem, Los Angeles, CA, U.S.A. Bovine serum albumin and ovalbumin, from Miles Laboratories, Kankakee, IL, U.S.A., and pig pancreatic trypsin and lysozyme, from Worthington Biochemical Co., Freehold, NJ, U.S.A., were also used as marker proteins for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The standard proteins, horse apoferritin, human γ -globulins, ovalbumin, albumin (bovine) and myoglobin (sperm whale) were supplied by Schwarz/Mann, Orangeburg, NY, U.S.A. Sephadex G-200 and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. The Whatman DE-52 ion exchanger was from H. Reeve Angel, Clifton, NJ, U.S.A. The Ampholine carrier ampholytes were obtained from USA LKB Instruments Inc., Rockville, MD, U.S.A. All other chemicals were of the highest purity available on the market.

Assay methods

Assay of rabbit testicular arylsulphatase A. Enzyme assays were performed by the method of Worwood *et al.* (1973) with the following modifications for maximum activity. Dilutions of enzyme were made with deionized water unless otherwise specified. The assay mixture consisted of 0.2 ml of 20 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate in 0.5 M-sodium acetate buffer, pH 5.2, and 0.2 ml of enzyme solution. The mixture was incubated for 10 min at 37°C in a shaking water bath and the reaction was terminated by the addition of 2.5 ml of 1 M-NaOH. The chromogen released, *p*-nitrocatechol, was measured by reading the E_{515} . The amount of *p*-nitrocatechol formed was determined from a standard curve. One unit of enzyme activity is the amount of enzyme that releases 1 μ mol of *p*-nitrocatechol from *p*-nitrocatechol sulphate/min under the assay conditions specified above. Another substrate, *p*-nitrophenyl sulphate (10 mM), was also used to test enzyme specificity. The reaction was terminated with 1 M- Na_2CO_3 and the released *p*-nitrophenol was measured by reading the E_{400} .

Other enzyme assays. Azocoll hydrolysis for general proteinase activity was determined by the method described in Calbiochem Document no. 3805. The esterase activity of acrosin (EC 3.4.21.10) was determined by the method of Schwert & Takenaka (1955), by using α -N-benzoyl-L-arginine ethyl ester hydrochloride. Hyaluronidase activity was determined colorimetrically by the method of Yang & Srivastava (1975b). β -Glucuronidase activity was determined by the method of Yang & Srivastava (1975b), by using phenolphthalein mono- β -glucuronide. β -Galactosidase activity was assayed by the method of Gatt & Rapport (1966) with *p*-nitrophenyl β -galactoside as substrate.

Protein determination. Biuret reagent was used for detecting high concentrations of protein by the method of Gornall *et al.* (1949), and protein in the range 10–200 μ g was determined by the method of Lowry *et al.* (1951), with crystallized bovine serum albumin (Miles Laboratories; fraction V, purity 99.6%, moisture 1.6%) as standard.

Purification of arylsulphatase A

Preparation of acetone-dried powder of rabbit testis and extraction of this powder with detergent. Rabbit testes devoid of epididymides were homogenized first at low speed for 5 min and then at high speed for 3 min in cold acetone (approx. -20° to -30°C) in a Waring blender. The mixture was filtered in a Büchner funnel and washed with 4 $\times$ 500 ml of cold acetone (at the same temperature). The residue was dried on filter paper.

The acetone-dried powder was suspended in 100 ml of 0.05 M-Tris/maleate buffer, pH 6.1, with 0.05 M- MgCl_2 , 0.7% Hyamine 2389, 1.4% Triton X-100 (both from Rohm and Haas, Philadelphia, PA, U.S.A.), 12.3 μ g of penicillin G/ml and 12.3 μ g of streptomycin/ml and with 2 drops of octan-2-ol, and stirred at 37°C for 1 h and then gently homogenized (two strokes up and down) with a Teflon-pestle homogenizer in a Potter-Elvehjem apparatus. The mixture was further stirred for 3 h at 37°C and then centrifuged at 27000g for 30 min in 4°C. To the supernatant, 200 ml of cold (-15°C) 95% (v/v) ethanol was added rapidly and the mixture stored overnight at -15°C . The next day the mixture was centrifuged at 4300g for 20 min (4°C) and the sediment was dissolved in 60 ml of deionized water and dialysed against 2 litres of 25 mM-Tris/HCl buffer, pH 7.4, in a cold-room (4°C) overnight with two changes of water. The non-diffusible material was centrifuged at 27000g for 20 min (4°C). The supernatant was freeze-dried and kept in the freezer (-15°C).

Step I, DEAE-cellulose chromatography. The freeze-dried material obtained by detergent treatment of rabbit testis acetone-dried powder was applied to a column (2.5 cm $\times$ 36 cm) of DE-52 DEAE-cellulose at 4°C pre-equilibrated with 0.02 M-Tris/HCl buffer, pH 7.4. The column was eluted with 650 ml of the same buffer and developed with a stepwise gradient of 0.2 M-NaCl and 0.5 M-NaCl in buffer (600 ml of each) at 40 ml/h. The second protein peak showing high constant arylsulphatase A activity across the peak was pooled and dialysed against deionized water with one change of water. The non-diffusible material was freeze-dried and kept at -15°C .

Step II, gel filtration on Sephadex G-200 column. The freeze-dried material obtained from the second peak of the DEAE-cellulose column (Step I) was dissolved in 0.05 M-sodium acetate buffer, pH 5.0,

and applied to a column (2.5cm×93cm; V_t = 454ml) of Sephadex G-200 pre-equilibrated with the buffer at 4°C. The void volume of the column was shown to be 169ml with Blue Dextran 2000. The column was eluted with 600ml of the buffer at 39ml/h. The arylsulphatase active fractions (5ml/tube) were pooled and dialysed against deionized water overnight. The non-diffusible material was concentrated with a Diaflo UM-10 filter to 32ml.

Step III, isoelectric-focusing column. The preparative isoelectric focusing was carried out by the procedure outlined by the manufacturer (LKB 8100 Ampholine Instruction Manual), by using an LKB electrofocusing column (LKB 8101, 110ml). The 1% Ampholine buffer (pH3–5, LKB 8151) in sucrose density gradient (53–0%, w/v) was used with the cathode as the upper electrode. The sucrose gradient was prepared with a gradient mixer and the light gradient solution contained 32ml of the arylsulphatase A-active solution obtained from gel filtration with Sephadex G-200. The electrode solutions were H_2SO_4 and NaOH for anode and cathode respectively. The power was maintained at 2–3W for 72h with starting voltage of 500V. Fractions (2ml) were collected at a rate of 25ml/h, and the pH of each fraction was checked at the same temperature (5°C) at which electrofocusing was performed.

Step IV, second gel filtration on Sephadex G-200. The active fractions from isoelectric-focusing columns were applied to a column (1.0cm×27.5cm, V_t = 21.6ml, V_0 = 8.2ml) of Sephadex G-200 at 4°C pre-equilibrated with 0.05M-sodium acetate buffer (pH5.6). The column was washed with 100ml of the buffer at 23ml/h. The fractions (2ml/tube) showing high constant arylsulphatase A activity were pooled and concentrated by Diaflo ultrafiltration. The concentrated enzyme fractions were stored in the refrigerator (4°C).

Other methods

Disc gel electrophoresis. Polyacrylamide-gel electrophoresis was performed at pH8.3 (current density 1.5mA/gel for 4h) on a 7.5% gel at room temperature (20°C) by the method of Brewer & Ashworth (1969). Bromophenol Blue was used as a tracking dye. The gels were stained with 0.04% Coomassie Brilliant Blue G-250 in 3.5% (v/v) $HClO_4$ as described by Reisner *et al.* (1975) and destained with water. The enzyme protein band on the unstained gel was identified by reaction with substrate (*p*-nitrocatechol sulphate) at room temperature and soaked in alkaline solution (1M-NaOH).

Estimation of molecular weight by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The molecular weight of the subunit was estimated as described by Weber & Osborn (1969). The final

preparation of enzyme was treated with 1% sodium dodecyl sulphate to neutralize the charges and 1% 2-mercaptoethanol to break all susceptible disulphide bonds. The samples were then subjected to electrophoresis in 4.3% polyacrylamide gels in the presence of sodium dodecyl sulphate at room temperature. The enzyme protein band was identified as described above. The mobility of the proteins was obtained by the equation of Weber & Osborn (1969). The molecular weight was plotted against mobility on semi-logarithmic paper. Bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000), pepsin (mol.wt. 35000), trypsin (mol.wt. 23300) and lysozyme (mol.wt. 14300) were used as references.

Estimation of molecular weight by gel filtration. The molecular weight of the enzyme was determined by chromatography on a column (2.5cm×94cm) of Sephadex G-200 by the procedure described in the Calibration Kit Instructional Manual from Pharmacia Fine Chemicals. The void volume of the column was determined with Blue Dextran 2000. The column was eluted with two buffers, 0.05M-sodium acetate buffer, pH5.0, and 0.05M-potassium phosphate buffer, pH7.1. Reference proteins were applied with both buffers, pH7.1 and 5.0. They were apoferritin (mol.wt. 480000), γ -globulin (mol.wt. 160000), albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000) and myoglobin (mol.wt. 17800).

Results

Purification of rabbit testicular arylsulphatase A

The starting material, the acetone-dried powder (2.2g of protein), was obtained from rabbit testes (42g blotted dry weight) which were kept frozen for 1 month. Threefold purification of the enzyme was achieved by treatment of acetone-dried powder (specific activity 0.09unit/mg of protein) with detergents. Overall purification was 430-fold from the acetone-dried powder to highly active enzyme obtained from Step IV. The purification of arylsulphatase A from rabbit testis is summarized in Table 1. Most of the arylsulphatase A activity was present in the supernatant after treatment with detergents. To remove detergents, the enzyme was precipitated with ethanol.

Most of the contaminant proteins were removed by the first step, DEAE-cellulose column chromatography (Fig. 1). Only the second peak (indicated by the arrow) and eluted by 0.2M-NaCl contained arylsulphatase A and a small amount of β -glucuronidase activities. Hyaluronidase and β -N-acetylglucosaminidase activities were present in the first peak. The proteinases hydrolysing Azocoll, a general proteinase substrate, appeared in the third peak eluted with 0.5M-NaCl.

The first attempt was made, by applying gel filtration on Sephadex G-200, to separate possible

Table 1. *Purification of arylsulphatase A from rabbit testis*

For Step III, protein and specific activity are approximate values, since the enzyme preparation is contaminated with Ampholine buffer.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
Detergent extraction	152	41	0.27	1	100
Step I DEAE-cellulose column	48	20	0.42	1.6	50
Step II Sephadex G-200 column	3.1	13.0	4.20	16	31
Step III Isoelectric-focusing column, pH 3-5	1.4	8.0	5.70	21	19
Step IV Sephadex G-200	0.2	7.6	38.1	141	18

Fig. 1. *DEAE-cellulose chromatography of detergent extracts, Step I*

Fractions (6.6 ml) were collected. See the Materials and Methods section for details. ●, E_{280} (protein); ○, E_{515} (arylsulphatase activity); ····, [NaCl].

arylsulphatases A and B, if these were present, as suggested by Worwood *et al.* (1973). This step II, gel filtration, also aimed at removing contaminant proteins. As shown in Fig. 2, only one major arylsulphatase A activity was found, with separation of low-molecular-weight contaminants. When the detergent extract was applied to Sephadex G-200, without prior fractionation on DEAE-cellulose under conditions identical with those reported by Worwood *et al.* (1973), the results were the same, i.e. only arylsulphatase A was obtained. The small β -glucuronidase contamination was removed from arylsulphatase A by Step II.

Further purification and evidence for one form of arylsulphatase A was obtained by isoelectric focusing. Before application of the sample obtained from the Sephadex G-200 column (Step II), the approximate

pI value was determined by using the sample obtained from Step I with Ampholine buffer, pH 3.5–10. Isoelectric focusing (Step III) was carried out with Ampholine buffer, pH 3–5, on the basis of the data obtained from the above trial. This gave better resolution compared with the broad-range-pH Ampholine. Arylsulphatase A from rabbit testis has pI 4.6 (Fig. 3). Major contaminant proteins even with slight differences in pI values could be separated by this step. Sucrose and Ampholine buffer (1%) used for the isoelectric focusing did not affect the enzyme activity. On the other hand, protein contents based on E_{280} readings of isoelectric-focusing fractions gave only approximate values, since the Ampholine buffer interfered. The samples obtained from isoelectric focusing showed slightly higher values for protein determined by the method of Lowry *et al.*

Fig. 2. Gel filtration on Sephadex G-200, Step II

Fractions (5 ml) were collected; for details of the method see the Materials and Methods section. ●, E_{280} (protein); ○, E_{515} (arylsulphatase A).

(1951). Therefore the data in Table 1 are approximate values.

Gel filtration with Sephadex G-200 (Step IV) was used for removing sucrose and Ampholine buffer as well as the possible contaminant proteins of different sizes. The fractions with constant specific activity indicated by arrows (Fig. 4) were pooled. Gel filtration (Step IV) resulted in a remarkable increase in specific activity of the enzyme. This step also eliminated substances interfering with protein determination and allowed measurement of correct protein contents.

Properties

Polyacrylamide-gel electrophoresis. The final preparation of arylsulphatase A obtained by Step IV showed one major band, with one minor band (Fig. 3). The major band on the left showed arylsulphatase A activity by activity staining with *p*-nitrocatechol sulphate.

pH optimum. As the incubation time is varied, the pH optima of rabbit testicular arylsulphatase A are

changed (Table 2). To ensure that the pH in assay solution remains unchanged, the buffer concentration was increased to four times that usual in the assay system. Several pH optima were observed for rabbit testicular arylsulphatase A with different times of incubation, as for spermatozoa acrosomal arylsulphatase (Yang & Srivastava, 1974a). Farooqui & Bachhawat (1972) reported that chicken brain arylsulphatase A showed similar shifts but had one major optimum for each time of incubation.

Temperature optimum. Arylsulphatase A from rabbit testis shows the maximum activity at 50°C when assayed with 20 mM *p*-nitrocatechol sulphate in 0.5 M-sodium acetate buffer (pH 4.8) with 30 min incubation, and in 0.5 M-sodium acetate buffer (pH 5.2) with 10 min incubation. A similar result was obtained by using 20 mM substrate in 0.5 M-sodium acetate buffer (pH 5.6) with 1 h incubation. Regardless of the pH and time of incubation, arylsulphatase A from rabbit testis showed temperature optimum at 50°C. Worwood *et al.* (1973) observed that arylsulphatase A from rat tissues showed the highest activity at around 25°C under the above conditions, whereas arylsulphatase B showed more activity at 50°C than at lower temperatures.

Effect of time of incubation on enzyme activity. The anomalous time-activity relationship with 4-nitrocatechol sulphate as substrate is a characteristic of many mammalian arylsulphatases A. The enzyme obtained from rabbit testis shows similar behaviour (Fig. 5). The rabbit testicular arylsulphatase A shows linear time-activity relationship with 0.25 mM-pyrophosphate, as do human liver arylsulphatase A (Baum *et al.*, 1958), chicken brain arylsulphatase A (Farooqui & Bachhawat, 1972) and arylsulphatase A from human urine (Stevens *et al.*, 1975). With other substrates such as 4-methylumbelliferyl sulphate, a linear time-activity relationship was observed with human brain arylsulphatase A (Harinath & Robins, 1971) and with human urine arylsulphatase A (Stevens *et al.*, 1975). Pyrophosphate significantly decreases the arylsulphatase A activity of rabbit testis. This seems to affect arylsulphatase B in the enzyme preparation, as reported by Baum *et al.* (1959). However, Cl^- ions (1.7 M), a specific inhibitor of arylsulphatase B, do not affect the activity of rabbit testicular arylsulphatase preparation, thereby indicating the absence of arylsulphatase B from the testicular enzyme, and therefore that the inhibition of arylsulphatase A activity of the testicular enzyme by pyrophosphate is real and unique as compared with A and B enzymes from other sources.

Inhibitors. Enzyme-inhibition studies were performed after preincubation of enzyme and inhibitor for 5 min. PO_4^{2-} and SO_3^{2-} were strong inhibitors of arylsulphatase A from rabbit testis. The K_i values obtained for inhibition with PO_4^{2-} and SO_3^{2-} were $6.3 \times 10^{-6} \text{ M}$ and $2.1 \times 10^{-4} \text{ M}$ respectively. SO_4^{2-}

Fig. 3. Profiles of preparative isoelectric-focusing column, Step III, and analytical gel electrophoresis of rabbit testicular arylsulphatase A obtained by the final step of gel filtration (Step IV)

Fractions (1.5 ml) were collected and pH values and enzyme activities were determined as described in the Materials and Methods section. The positive (+) and negative (–) signs indicate poles of both disc gel electrophoresis and the isoelectric-focusing column. The major protein band, indicated by arrow, showed arylsulphatase activity, and the minor contamination originated from the large peak near the arylsulphatase-active peak on the chromatogram; 60 μ g of protein was applied to the gel. ●, E_{280} (protein); ○, E_{515} (arylsulphatase A); ····, pH gradient.

slightly inhibited the enzyme, with K_i 3.1×10^{-2} M. Similar results were observed for chicken brain arylsulphatase A (Farooqui & Bachhawat, 1972). NaCl or KCl (both 50 mM) did not inhibit arylsulphatase A from rabbit testis. A previous report on the acrosomal enzyme indicated that NaCl, KCl and SO_4^{2-} do not affect enzyme activity (Yang & Srivastava, 1974a). Both rabbit spermatozoa acrosomal arylsulphatases and testicular arylsulphatase A were initially inhibited when assayed with a 10 min reaction time, even without preincubation of enzyme and inhibitor. However, inhibition was quickly decreased by in-

creasing the reaction time, and finally, the enzymes were not affected by SO_4^{2-} after 1 h of incubation. Arylsulphatase A from rabbit testis was slightly inhibited by Ag^+ , as was the enzyme from other sources, such as ox and human brain (Bleszynski & Leznicki, 1967; Harinath & Robins, 1971). α -Chlorohydrin, a chemical male sterilant in several mammals, inhibited testicular arylsulphatase A as well as spermatozoa acrosomal arylsulphatases (Yang & Srivastava, 1976).

Specificity and kinetic parameters. The rabbit testicular arylsulphatase A was inactive towards

Fig. 4. Second gel filtration on Sephadex G-200 column, Step IV

Fractions (2.0ml) were collected; see the Materials and Methods section for details. ●, E_{280} (protein); ○, E_{515} (arylsulphatase A).

Fig. 5. Time-activity curve for rabbit testicular arylsulphatase A

The enzyme used for this experiment was obtained from the active fractions from the DEAE-cellulose column and 14 μ g of enzyme protein in a total volume of 0.4 ml of assay mixture was used for each assay. The assay was performed as described in the text: ●, without sodium pyrophosphate; ○, with 0.25 mM-sodium pyrophosphate.

Table 2. pH optimum as a function of time of incubation

The enzyme used for this experiment was obtained from the active fractions from the DEAE-cellulose column and 25 μ g of enzyme protein was used in a total volume of 0.4 ml for each assay. The 0.5M-sodium acetate solution was adjusted to different pH values with 0.5M-acetic acid or 0.5M-NaOH. The assay was performed as described in the Materials and Methods section.

Incubation time (min)	pH optimum
5	5.2
20	5.0
30	4.8
90	4.9

20mM-*p*-nitrophenyl sulphate at pH 4.8 and 5.2. The kinetic parameters of rabbit testicular arylsulphatase A with *p*-nitrocatechol sulphate as substrate were

obtained by a double-reciprocal plot. The K_m and V_{max} were 4.1 mM and 80 nmol/min at 37°C. Product inhibition was observed when the low concentration of enzyme was used. Yamato *et al.* (1974) reported that arylsulphatase A from boar testis has activity towards natural substrates such as 35 S-labelled sulphatide and a sulphated glyceroglycolipid present in boar testis and spermatozoa.

Estimation of molecular weight. A Sephadex G-200 column ($V_t = 461$ ml) was used to determine the molecular weight of the enzyme at two different pH values. The void volume of the column was 184 ml, as determined with Blue Dextran 2000. The selectivity curve (plot of K_{av} values versus the logarithm of molecular weight) for standard proteins and the enzyme at two different pH values is shown in Fig. 6. Rabbit testicular arylsulphatase A at pH 7.1 was eluted after γ -globulin, suggesting a mol.wt. of 110000. However, enzyme that was kept in acetate buffer, pH 5.0, overnight gave an approximate

Fig. 6. Selectivity curve (plot of K_{av} versus the logarithm of molecular weight) for standard protein and arylsulphatase A from rabbit testis at pH 7.1 and 5.0 chromatographed on Sephadex G-200 (superfine grade)

Experimental details are given in the text. K_{av} for each protein is obtained from the equation

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

V_e , elution volume for the protein; V_0 , elution volume for Blue Dextran 2000; V_t , total bed volume. Standards used were: (1) apoferritin, mol.wt. 480000; (2) arylsulphatase A (pH 5.0), mol.wt. 220000; (3) γ -globulin, mol.wt. 160000; (4) arylsulphatase A (pH 7.1), mol.wt. 110000; (5) albumin, mol.wt. 67000; (6) ovalbumin, mol.wt. 45000; (7) myoglobin, mol.wt. 17800.

mol.wt. of 220000. The elution of reference proteins at two different pH values was exactly the same. The enzyme was partially inhibited by the potassium phosphate buffer used in the gel for determining the mol.wt. of the enzyme at pH 7.1.

The subunit molecular weight of rabbit testicular arylsulphatase A after treatment with sodium dodecyl sulphate and mercaptoethanol was approx. 55000, as shown by the migration of the enzyme and standard proteins. The enzyme band was identified by activity staining with substrate. Thus arylsulphatase A from rabbit testis consists of two identical subunits. Similar-sized subunits have been found for ox liver (Roy & Jerfy, 1970) and for human urine enzymes (Stevens *et al.*, 1975).

This indicates that rabbit testicular arylsulphatase A exists as a dimer of mol.wt. of 110000 at pH 7.0. At pH 5.0, rabbit testicular arylsulphatase A exists as a tetramer of approx. mol.wt. 220000. Arylsulphatase A from ox liver (Nichol & Roy, 1965) and rat liver (Worwood *et al.*, 1973) exists as a tetramer of approx. mol.wt. 400000 at pH 5.0.

Discussion

This is the first report on purification and characterization of rabbit testicular arylsulphatase. Experiments to identify isoenzyme activities in testicular arylsulphatase preparations obtained at different steps of purification and isoelectric focusing of the enzyme preparations conclusively showed that rabbit testicular arylsulphatase exists only in one form, which resembles mainly arylsulphatase A of other tissues with respect to its substrate specificity, anomalous time-activity relationship, molecular weight and inhibition by PO_4^{2-} , SO_3^{2-} , SO_4^{2-} and Ag^+ ions.

The isoelectric point of rabbit testicular arylsulphatase A is about the same as that of arylsulphatase A of human liver (Neuwelt *et al.*, 1972), urine (Stevens *et al.*, 1973) and placenta (Gniot-Szulżycka, 1974). Arylsulphatases from kangaroo liver (Roy, 1971) and ox liver arylsulphatase A (Nichol & Roy, 1964) have different isoelectric points, 5.1–5.4 and 3.4 respectively. Ox liver arylsulphatase B has pI 8.2 (Allen & Roy, 1968). Stevens *et al.* (1973) observed two forms of arylsulphatase A, $A\alpha$ and $A\beta$, from human urine, but there is no indication of minor forms of enzyme in rabbit testicular extracts.

The specific activity of purified arylsulphatases from rabbit spermatozoa acrosomes was erroneously expressed as units/mg of protein by Yang & Srivastava (1974a) instead of the correct value of munits/mg of protein. Yamato *et al.* (1974) purified arylsulphatase A from boar testis, but the specific activity of their purified enzyme was much less than that of the purified enzyme reported here. One major and several minor pH optima for rabbit testicular arylsulphatase A were observed, depending on the time of incubation. Arylsulphatases obtained from rabbit spermatozoa acrosomes showed three optima, at pH 4.8, 5.6 and 6.0 (Yang & Srivastava, 1974a). Two major optima at pH 4.4 and 5.2 with different times of incubation have been observed for human liver arylsulphatase A (Baum *et al.*, 1958) and chicken brain arylsulphatase A has optima at pH 5.0 and 5.5 (Farooqui & Bachhawat, 1972). Yamato *et al.* (1974) reported one optimum, pH 4.5, for boar testicular arylsulphatase A. With a different time of incubation, Harinath & Robins (1971) observed only one optimum, pH 5.7, for human brain arylsulphatase A with 4-methylumbelliferone sulphate as substrate. The two forms of arylsulphatase A, $A\alpha$ and $A\beta$, from human urine had wide optima between pH 5.7 and 6.0 (Stevens *et al.*, 1973).

Like rabbit spermatozoa acrosomal arylsulphatases (Yang & Srivastava, 1974a), rabbit testicular arylsulphatase A showed the highest activity at 50°C. The characteristic temperature optimum of the enzyme was significantly different from that of rat tissues (Worwood *et al.*, 1973). The rabbit testicular enzyme shares the temperature optimum of rat tissue

arylsulphatase B. Rat tissue arylsulphatase A has a much lower temperature optimum, i.e. about 25°C.

Rabbit testicular arylsulphatase A was inhibited by PO_4^{2-} , SO_3^{2-} and SO_4^{2-} ions, but rabbit acrosomal arylsulphatases were not inhibited by SO_4^{2-} (Yang & Srivastava, 1974a). The differences in SO_4^{2-} -ion inhibition of the acrosomal and testicular arylsulphatases were due to the assay procedures applied, as explained in the Results section. Both enzymes were not affected by SO_4^{2-} ion with the assay procedure described in the present paper, involving an extended time of incubation. Thus the properties of both arylsulphatases from rabbit spermatozoa acrosomes and arylsulphatase A from rabbit testis are essentially identical. Roy's (1958) assay system used in the earlier report (Yang & Srivastava, 1974a) to determine arylsulphatases A and B was inadequate, as these forms of enzymes can only be determined properly after separation, as suggested by Worwood *et al.* (1973).

Rabbit testicular arylsulphatase A exists as a dimer at neutral pH; however, the enzyme is a monomer at pH 5, showing maximum activity at this pH. The enzyme consists of two identical subunits. Arylsulphatase B of other species such as rat tissue (Worwood *et al.*, 1973) and ox liver (Allen & Roy, 1968) is reported to have a much lower molecular weight than that of the subunit of rabbit testicular arylsulphatase A. Similar subunit size was observed in human urine arylsulphatase A (Stevens *et al.*, 1975). Both ox liver (Nichol & Roy, 1965) and rat tissue arylsulphatases (Worwood *et al.*, 1973) exist as a tetramer at pH 5.0. Human placenta arylsulphatase A exists as a monomeric form at pH 5.0 and 7.5, as estimated by thin-layer gel filtration (Gniot-Szulzycka, 1974). Two arylsulphatases from human brain, that are absorbed and excluded on DEAE-cellulose, have apparent mol.wts. of 109000 and 51300 respectively (Harinath & Robins, 1971). All of the rabbit testicular arylsulphatase activity was adsorbed on DEAE-cellulose under the same conditions.

The possible role of the enzyme in the fertilization process is not clear. As the cumulus, corona and the zona pellucida of the ovum consist of mucopolysaccharides, it is suggested that this enzyme may facilitate the spermatozoa to penetrate the ovum in combination with other glycosidases and proteinases (Yang & Srivastava, 1974a). A natural substrate of the enzyme, named 'seminolipid', was obtained from boar testis and spermatozoa (Ishizuka *et al.*, 1973). Arylsulphatase A purified from boar testis showed activity toward the seminolipid and labelled sulphatide (Yamato *et al.*, 1974). This indicates that the enzyme might have possible functions in spermatozoa maturation, capacitation or the acrosome reaction.

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