Purification and Properties of Arylsulphatase A from Chicken Brain

By A. A. FAROOQUI and B. K. BACHHAWAT
*Neurochemistry Laboratory, Department of Neurological Sciences,
Christian Medical College Hospital, Vellore 4, S. India*

(Received 1 October 1971)

1. Chicken brain arylsulphatase A was purified 2000-fold, with overall recovery 14%, by using ammonium sulphate fractionation, ethanol precipitation, Sephadex G-200 gel filtration and DEAE-Sephadex column chromatography. 2. The purified preparation was free from β -glucuronidase, β -galactosidase, acid phosphatase, inorganic pyrophosphatase and adenosine 3'-phosphate 5'-sulphatophosphate sulphohydrolase activities. 3. Polyacrylamide-gel electrophoresis indicated that the purified preparation was not homogeneous. 4. Chicken brain arylsulphatase was markedly inhibited by carbonyl reagents in the presence of traces of Cu^{2+} in the system. Other metal ions such as Fe^{2+} and Zn^{2+} , were inactive. 5. Ascorbic acid alone had no effect on enzyme activity but enhances the inhibition by Cu^{2+} . 6. Chicken brain arylsulphatase A resembled arylsulphatase A of other animal species in its kinetic properties such as K_m value, anomalous time-activity relationship and the inhibitory effect of phosphate, sulphite and sulphate ions. However, its electrophoretic mobility, behaviour under zinc acetate fractionation and stimulation by Ag^+ were similar to arylsulphatase B of other animal species. Thus, this enzyme did not correspond to either arylsulphatase A or arylsulphatase B but properties of both. 7. The purified enzyme preparation can degrade cerebroside 3-sulphate.

Lysosomal arylsulphatases A and B (arylsulphate sulphohydrolase, EC 3.1.6.1) have been found in all mammalian tissues investigated. The two enzymes are distinguished by their different pH optima, substrate affinities, anomalous kinetics and electrophoretic mobilities. Arylsulphatase A and B had been extensively purified from various mammalian tissues (Nichol & Roy, 1964; Allen & Roy, 1968; Bleszynski *et al.*, 1969; Szulzycka & Komoszynski, 1970; Roy, 1971; Harinath & Robins, 1971). Ignorance about the natural substrate made it difficult to assign any physiological role to these enzymes. A significant decrease in arylsulphatase A activity in the genetic disorder metachromatic leucodystrophy, which is characterized by abnormal accumulation of cerebroside 3-sulphate, was observed by Austin *et al.* (1963, 1964). Mehl & Jatzkewitz (1968) have reported that arylsulphatase A from pig kidney in the presence of a heat-stable factor can degrade cerebroside 3-sulphate. The role of arylsulphatase B is obscure. However, it has been suggested (Farooqui & Bachhawat, 1971) that arylsulphatase B may have a role in mucopolysaccharide metabolism. Work by Farooqui & Bachhawat (1971) has indicated the differences in proportions and physicochemical properties of brain arylsulphatases in various animal species. Chicken brain arylsulphatase resembles arylsulphatase A of other species in its kinetic properties such as K_m value, anomalous time-activity relationship and the inhibitory effect of sulphite, phosphate and sulphate ions. However, its electrophoretic mobility and be-

haviour under zinc acetate fractionation it is similar to the arylsulphatase B of other species.

In the present paper we describe the purification and properties of arylsulphatase A from chicken brain. Some properties of this partially purified arylsulphatase A are compared with those of the human brain arylsulphatase A. Results are presented to show that chicken brain arylsulphatase A can degrade cerebroside 3-sulphate.

Experimental

Materials

The dipotassium salt of 4-nitrocatechol sulphate and phenolphthalein β -glucuronide were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Aquacide I (polyethylene glycol) was obtained from Calbiochem, Los Angeles, Calif., U.S.A. *p*-Nitrophenyl β -galactoside was purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Sephadex G-200 and DEAE-Sephadex A-50 were purchased from Pharmacia, Uppsala, Sweden. [^{35}S]Sulphate was obtained from the Atomic Energy Establishment, Trombay, India, and all chemicals used were of analytical grade.

Methods

Assay of chicken brain arylsulphatase A. The enzyme was assayed by measuring the production of 4-nitrocatechol from 4-nitrocatechol sulphate. The

assay system consisted of 0.2M-sodium acetate buffer, pH 5.0, 6mM-nitrocatechol sulphate and enzyme in a total volume of 0.1 ml. The incubation was done at 37°C for 20 min, then the reaction was stopped by addition of 2.9 ml of 0.11 M-NaOH. The nitrocatechol that formed was measured in a Klett-Summerson colorimeter by using the 50 filter. The amount of 4-nitrocatechol formed was calculated from a standard curve.

Isolation of cerebroside 3-[³⁵S]sulphate. The rats (17 days old) were injected intraperitoneally with carrier-free Na₂³⁵SO₄ (20 µCi/g body wt.) and killed after 24 h. The brains were removed and homogenized in chloroform-methanol (2:1, v/v) (19 ml/g of wet tissue) in a Potter-Elvehjem homogenizer for 5 min and the homogenate was filtered. The filtrate was partitioned with 0.2 vol. of 0.74M-KCl and then the lower phase was washed twice with equal volumes of theoretical upper phase by the method of Folch *et al.* (1957). The lower phase was concentrated to dryness under vacuum and passed through a Florisil column by the method of Rouser *et al.* (1967). The cerebroside and sulphatide were eluted with chloroform-methanol (7:3, v/v) and concentrated under vacuum at 40°C. The cerebroside and sulphatide were separated by DEAE-cellulose acetate chromatography by the method of Rouser *et al.* (1961). The specific radioactivity of the isolated cerebroside 3-sulphate was 3.7×10^6 c.p.m./mg.

Protein determination. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

β-Glucuronidase. The enzyme was assayed by the method of Plapp & Cole (1966) with phenolphthalein β-glucuronide as substrate.

β-Galactosidase. The enzyme was assayed by the method of Gatt & Rapport (1966) with *p*-nitrophenyl β-galactoside as substrate.

Acid phosphatase. The enzyme was assayed by the method of Austin *et al.* (1963).

Inorganic pyrophosphatase. The enzyme activity was determined by the method of Sussman & Laga (1968) with inorganic pyrophosphate as substrate; the P_i released was measured by the method of Fiske & SubbaRow (1925).

Adenosine 3'-phosphate 5'-sulphatophosphate sulphohydrolase. The enzyme was assayed by the method of Balasubramanian & Bachhawat (1962), with adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate.

Polyacrylamide-gel electrophoresis. This was done as described by Farooqui & Bachhawat (1971) in 0.03M-sodium barbitone buffer, pH 8.0, for 15 h at 8 mA/tube in a Buchler instrument. Enzyme was eluted from the gel as described by Farooqui & Bachhawat (1971). The proteins were stained by Coomassie Brilliant Blue (Chrambach *et al.*, 1967).

Preparation of enzyme. All operations were done at 4°C unless otherwise mentioned.

Frozen chicken brain (500 g) was homogenized with 1 litre of 0.2M-sodium acetate buffer, pH 5.0, in a Waring Blendor for 2 min. The homogenate (1700 ml) was centrifuged at 12000g for 30 min and the supernatant (750 ml) was adjusted to 1.64M by adding 4.1M-(NH₄)₂SO₄. The precipitate that formed was dissolved in 0.02M-sodium acetate buffer, pH 5.0, and dialysed for 8 h against 500 vol. of 0.02M-tris-HCl buffer, pH 7.4. After dialysis the solution was stored overnight at 0°C and insoluble material was removed by centrifugation at 12000g for 30 min. The supernatant (62 ml) was subjected to ethanol precipitation. Aldehyde-free ethanol was added dropwise (1 ml/min) with constant mechanical stirring, to a final concentration of 40% (v/v). After 10 min the solution was centrifuged at 12000g for 30 min. The precipitate was discarded and the supernatant was dialysed against 100 vol. of 0.02M-tris-HCl buffer, pH 7.4, for 8 h. After dialysis the solution (180 ml) was adjusted to 40% saturation by addition of solid (NH₄)₂SO₄. The precipitate obtained after centrifugation for 30 min at 12000g was dissolved in 0.02M-sodium acetate buffer, pH 5.0, and was dialysed overnight against 100 vol. of 0.02M-tris-HCl buffer, pH 7.4.

The dialysed preparation (8 ml) was applied to a Sephadex G-200 column (1.28 cm × 38 cm) that was equilibrated with 0.02M-tris-HCl buffer, pH 7.4. The column was eluted in 5 ml fractions with the same buffer at a flow rate of 15 ml/h. The fractions that contained the enzyme were pooled and made 90% saturated with (NH₄)₂SO₄. The precipitate that was obtained after centrifugation was dissolved in the minimum amount of 0.02M-sodium acetate buffer, pH 5.0 and was dialysed overnight against 500 vol. of 0.02M-tris-HCl buffer, pH 7.4.

The clear solution (7 ml) was then applied to a column (0.5 cm × 7 cm) of DEAE-Sephadex A-50 equilibrated with 0.02M-tris-HCl buffer, pH 7.4. The column was washed with this buffer, at a flow rate of 12 ml/h. The washings did not contain any enzyme activity and were discarded. Stepwise elution was done with a gradient of 0.05–0.1M-NaCl in 0.02M-tris-HCl buffer, pH 7.4. Most of the enzyme activity was eluted in the 0.1M-NaCl fraction. This fraction was used to study the enzyme properties.

Concentration of enzyme by Aquacide I. The fraction eluted by 0.1M-NaCl from the DEAE-Sephadex column was concentrated by placing it in a dialysis bag, which was covered by Aquacide and kept at 4–6°C for 8 h. By this procedure tenfold concentration of the enzyme protein was achieved without any appreciable loss of enzyme activity. The concentrated enzyme was dialysed against 500 vol. of 0.02M-tris-HCl buffer, pH 7.4, for 6 h and was used to study the degradation of cerebroside 3-sulphate.

Purification of arylsulphatase A from human brain. Arylsulphatase A of human brain was purified by the method of Balasubramanian & Bachhawat (1963).

Results

Chicken brain arylsulphatase A was purified 2000-fold (Table 1) with an overall recovery of 14% by the above procedure. There was a considerable increase in total enzyme activity when the 12000g supernatant was fractionated with 1.64M-(NH₄)₂SO₄. This might be caused by removal of an endogenous inhibitor, because when 12000g supernatant that had been dialysed was added to the purified enzyme, there was about 40% inhibition of enzyme activity. For a reproducible result with ethanol fractionation it was necessary to maintain the temperature at 4–6°C.

The elution profile of chicken brain arylsulphatase A through a Sephadex G-200 column is shown in Fig. 1. The enzyme was eluted from the column as a single symmetrical peak, which did not coincide with the main protein peak. Fig. 2 shows the elution profile of the same enzyme through a DEAE-Sephadex A-50 column. Most of the enzyme activity was eluted in the 0.1M-NaCl fraction as a single peak. The puri-

fied enzyme preparation was completely free from β-glucuronidase, β-galactosidase, acid phosphatase, inorganic pyrophosphatase and adenosine 3'-phosphate 5'-sulphatophosphate sulphydrolase activity and could be stored for 2 months at 0°C without any appreciable loss of enzyme activity.

The purity of this chicken brain arylsulphatase A as judged by polyacrylamide-gel electrophoresis (Fig. 3) indicated that the preparation was not homogeneous. It had two protein bands. The arylsulphatase activity in various segments of the gel was assayed after being eluted and the activity was located in the major protein band, whereas the minor protein band had no enzyme activity. In an earlier paper we reported that the electrophoretic mobility of chicken brain arylsulphatase A was similar to that of arylsulphatase B of other animal species. The electrophoretic mobility of purified preparation was the same as reported previously (3cm) (Farooqui & Bachhawat, 1971).

Table 1. Purification of arylsulphatase A from chicken brain

The homogenate and 12000g supernatant were assayed after 8 h dialysis against 0.02M-tris-HCl buffer, pH7.4. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of 4-nitrocatechol from 4-nitrocatechol sulphate in 20 min under the usual assay conditions.

Enzyme fraction	Total volume (ml)	Total protein (mg)	Total enzyme (units)	Specific activity	Recovery (%)
Homogenate	1700	54400	816	0.015	100
12000g supernatant	750	3750	468	0.12	57
Ammonium sulphate (1.64M)	62	446	1023	2.3	125
Ethanol supernatant	180	300	518	1.7	63
Ammonium sulphate (40%)	16	198	614	3.1	72
Sephadex G-200 gel filtration	7	36	403	11.2	49
DEAE-Sephadex fraction	32	3.84	115	30.0	14

Fig. 1. Sephadex G-200 elution profile of chicken brain arylsulphatase A

Fractions (5ml) were collected. The experimental details and analysis of fractions for the protein and enzyme activity are described in the text. ●, Protein; ○, arylsulphatase A activity.

Fig. 2. DEAE-Sephadex A-50 elution profile of chicken brain arylsulphatase A

Fractions (2.0ml) were collected and analysed for enzyme activity as described in the text.

Properties of chicken brain arylsulphatase A

pH optimum. The enzyme had a pH optimum at 5.5 in sodium acetate buffer (Fig. 4) when incubated for 5 min but when the time of incubation was increased to 30 min the pH optimum shifted from 5.5 to 5.0 as shown in Table 2. Baum *et al.* (1958) reported that after incubation for 10 min the human liver arylsulphatase A showed optimum activity at two pH values, which over long incubation periods or at higher enzyme concentration merged into a single pH optimum. Harinath & Robins (1971) found only one pH optimum for the human brain arylsulphatase A with 4-methylumbelliferone sulphate as a substrate. Phosphate and sulphite ions did not shift the pH optimum after incubation for 5 min (Fig. 4).

Effect of time of incubation on enzyme activity. The relation between enzyme activity and time of incubation at various pH values is shown in Fig. 5. In no case was the activity proportional to the time of incubation over the whole incubation period. The pattern of the time-activity curve (Fig. 6) of this enzyme was similar to that of other animal species (Farooqui & Bachhawat, 1971). Harinath & Robins (1971) did not observe any anomalous time-activity relationship for human brain arylsulphatase A with 4-methylumbelliferone sulphate substrate. Chicken brain arylsulphatase A showed a linear time-activity relationship in the presence of pyrophosphate, as reported by Baum *et al.* (1958) for the human liver arylsulphatase A.

Effect of sulphite, phosphate and sulphate on enzyme activity. The enzyme activity was markedly inhibited by sulphite, phosphate and sulphate ions. The K_i values for sulphite, phosphate and sulphate ions were $1.2 \times 10^{-5} M$, $0.48 \times 10^{-4} M$ and $0.92 \times 10^{-2} M$ respectively. In all cases the inhibition was competitive.

Fig. 3. Electrophoretic mobility of chicken brain arylsulphatase A

A 7.5% polyacrylamide gel in a 17 cm $\times$ 1 cm tube was used. The conditions for electrophoresis were as described in the text. The major protein band, indicated by the arrow, contained the enzyme activity.

Table 2. pH optimum as a function of time of incubation for chicken brain arylsulphatase A

The assay procedure is described in the Experimental section. The 0.2 M-sodium acetate buffer was adjusted to the indicated pH values, and 10 μ g of enzyme protein was used in each assay.

Time (min)	pH optimum
5	5.5
20	5.5
30	5.0
90	5.0

Harinath & Robins (1971) found, with 4-methylumbelliferone sulphate as substrate, that the arylsulphatase A of human brain was competitively inhibited by sulphate ions.

Effect of cyanide, fluoride and chloride ions on enzyme activity. Potassium cyanide (10 mM) caused about 16% inhibition whereas sodium fluoride at

Fig. 4. pH-activity curve of chicken brain arylsulphatase A with phosphate and sulphite ions

The assay mixture consisted of 0.2M-sodium acetate buffer of various pH values, 6mM-nitrocatechol sulphate and 4.8 μ g of enzyme protein in a total volume of 0.1 ml. After incubation for 5 min the reaction was terminated and the 4-nitrocatechol formed was determined as described in the text. $\circ$, No addition; $\square$, 0.25 mM-phosphate; $\bullet$, 25 μ M-sulphite.

this concentration caused 67% inhibition of enzyme activity (Table 3). Balasubramanian & Bachhawat (1963) found that fluoride ions caused marked inhibition compared with cyanide ions with human brain arylsulphatase A. Sodium chloride (10mM) had no effect on enzyme activity.

Effect of carbonyl reagent, ascorbate and metal ions on enzyme activity. Like ox liver arylsulphatase A (Roy, 1970) both chicken and human brain arylsulphatase A were inhibited by carbonyl reagents (Table 4). The inhibition was more marked in the presence of 0.02mM- Cu^{2+} (Table 5). Other metal ions such as Fe^{2+} and Zn^{2+} at 0.2mM were not effective. In the absence of carbonyl reagents, Cu^{2+} inhibited the enzyme activity at a very high concentration (2mM). Other metal ions such as Fe^{2+} , Zn^{2+} and Co^{2+} did not affect the enzyme activity at 0.02–2mM. Bleszynski & Leznicki (1967) and Harinath & Robins (1971) reported that silver nitrate (0.25mM) produced 96% inhibition of arylsulphatase A whereas it stimulated the activity of arylsulphatase B. With chicken brain arylsulphatase A silver nitrate (0.025–1mM) did not inhibit enzyme activity and a low concentration (0.025mM) stimulated enzyme activity

Fig. 5. Nitro catechol formation as a function of time at various pH values

The assay mixture consisted of 0.2M-sodium acetate buffer of various pH values, 6mM-nitrocatechol sulphate and 10 μ g of enzyme protein in a total volume of 0.1 ml. The reaction was terminated and the 4-nitrocatechol formed was determined as described in the text. $\bullet$, pH4.0; $\circ$, pH4.5; $\square$, pH5.0; $\blacksquare$, pH5.5.

by 20%. Ascorbate did not inhibit the activity of either chicken or human brain arylsulphatase A. The inhibition by Cu^{2+} was enhanced by ascorbate. Other metal ions such as Fe^{2+} and Zn^{2+} (0.02–0.2mM) did not affect the enzyme activity even in the presence of ascorbate.

Effect of cysteine and p-hydroxymercuribenzoate on enzyme activity. Cysteine did not have any appreciable effect on the enzyme activity. p-Hydroxymercuribenzoate (1mM) and N-ethylmaleimide (2mM) did not affect the enzyme activity at pH 5.5, which was the optimum pH for the enzyme activity, but at pH 5.0 considerable inhibition was observed (Table 6). The inhibition by p-hydroxymercuribenzoate at pH 5.0 was reversed by cysteine. These findings agree with those of Jerfy & Roy (1969) who reported that ox liver enzyme at high pH values was not inhibited by p-chloromercuribenzoate but at lower pH values it was, and this inhibition could be reversed by cysteine. Balasubramanian & Bachhawat (1963) found that p-chloromercuribenzoate could inhibit arylsulphatase A of human brain, but this inhibition could not be reversed by cysteine at pH 4.5. Bleszynski & Leznicki (1967) found that ox brain arylsulphatase A was markedly inhibited by p-chloromercuribenzoate but this inhibition could not

be reversed by glutathione. Harinath & Robins (1971) observed 77% inhibition of human brain arylsulphatase A by 0.1 mM-*p*-chloromercuribenzoate. These

Fig. 6. Time-activity curve for chicken brain arylsulphatase A with and without pyrophosphate

The assay mixture consisted of 0.2M-sodium acetate buffer, pH 5.5, 6mM-nitrocatechol sulphate and 2.4 μg of enzyme protein in a total volume of 0.1 ml. The reaction was terminated and the 4-nitrocatechol formed was determined as described in the text. ○, No addition; ●, 0.25 mM-sodium pyrophosphate.

authors did not see if thiol reagents restored enzyme activity.

Specificity of the enzyme. The chicken brain arylsulphatase A was inactive towards 4-hydroxy 5-nitrophenyl sulphate between pH 4.0 and pH 8.0. The enzyme showed no adenosine 3'-phosphate 5'-sulphatophosphate sulphohydrolase activity under the assay conditions described by Balasubramanian & Bachhawat (1962). However, this enzyme could degrade cerebroside 3-sulphate at pH 4.0 (Table 7).

Table 3. Effect of cyanide, fluoride and chloride ions on chicken brain arylsulphatase A

The purified chicken brain arylsulphatase A was kept at room temperature for 1 h in 0.2M-sodium acetate buffer, pH 5.5, with the indicated concentrations of anions, with occasional shaking. The reaction was started by the addition of nitrocatechol sulphate (6mM) in a final volume of 0.1 ml. The tubes were incubated for 5 min at 37°C; the reaction was terminated and the 4-nitrocatechol formed was determined as described in the text; 4.8 μg of enzyme protein was used. A control reaction mixture containing all the components except anions was treated in the same way.

Component added	Concentration (mM)	Nitrocatechol formed (nmol)
Control	—	45
Cyanide	5	39
	10	38
Fluoride	5	18
	10	15
Chloride	5	42
	10	35

Table 4. Effect of carbonyl reagents on chicken and human brain arylsulphatase A

The purified chicken brain arylsulphatase A was treated with reagents and its activity assayed as described in Table 3. The purified human brain arylsulphatase A was treated similarly, except that it was kept with the reagent at pH 5.0 for 30 min; 55.6 μg of enzyme protein was used, and the incubation was for 20 min at 37°C. The control reaction mixtures contained all the components except carbonyl reagent, and 4-nitrocatechol sulphate was added after 1 h (chicken) and 30 min (human) under similar conditions.

Carbonyl reagents	Concentration	Nitrocatechol formed (nmol)	
		Chicken arylsulphatase A	Human arylsulphatase A
Control	—	45	45
Hydroxylamine	1 mM	28	20
	2 mM	20	15
Phenylhydrazine	1 mM	13	45
	2 mM	4	24
Hydrazine	0.5 mM	34	—
	1 mM	23	—

Table 5. *Effect of copper, carbonyl reagent and ascorbate on chicken and human brain arylsulphatase A*

The enzyme was kept at room temperature in sodium acetate buffer, pH 5.5 (chicken) and pH 5.0 (human), with Cu^{2+} , carbonyl reagent and ascorbate; it was assayed as described in Table 4; 4.8 μg (chicken) and 55.6 μg (human) enzyme protein was used. In the control reaction mixture none of the component was added.

Component added	Concentration (mM)	Nitrocatechol formed (nmol)	
		Chicken arylsulphatase A	Human arylsulphatase A
Control	—	45	45
Cu^{2+}	0.05	46	—
	2	5	33
Phenylhydrazine	0.5	28	45
Phenylhydrazine + Cu^{2+}	0.5 } 0.02 }	20	6
Hydroxylamine	1	30	20
Hydroxylamine + Cu^{2+}	1 } 0.02 }	6	14
Ascorbate	1	40	48
Ascorbate + Cu^{2+}	1 } 0.02 }	5	36

Table 6. *Effect of cysteine, p-hydroxymercuribenzoate and N-ethylmaleimide on chicken brain arylsulphatase A*

The purified chicken brain arylsulphatase A was kept at room temperature for 1 h in 0.2M-sodium acetate buffer, pH 5.0 and 5.5, with required concentration of component with occasional shaking. In studies on the reversal of p-hydroxymercuribenzoate inhibition by cysteine, the neutralized cysteine was added just before starting the reaction by the addition of nitrocatechol sulphate (6mM) in a final volume of 0.1 ml. Other conditions were as described in Table 3.

Component added	Concentration (mM)	Nitrocatechol formed (nmol)	
		pH 5.0	pH 5.5
Control	—	24	38
Cysteine	1	—	40
	2.5	—	33
p-Hydroxymercuribenzoate	0.5	16	41
	1	8.4	33
p-Hydroxymercuribenzoate + cysteine	0.5 } 10 }	24	—
p-Hydroxymercuribenzoate + cysteine	1 } 10 }	24	—
N-Ethylmaleimide	1	24	38
	2	18	38

The [^{35}S]sulphate liberated was identified by paper chromatography. The reaction product as well as standard [^{35}S]sulphate had R_F 0.28 and the undegraded cerebroside 3-sulphate moved with the solvent front.

As mentioned above the purified enzyme had two protein bands and the major band had arylsulphatase activity. To check whether cerebroside sulphatase activity resided in the same band or not, this band was cut out from several gels, the eluted protein was

Table 7. *Degradation of cerebroside 3-sulphate by chicken brain arylsulphatase A*

The reaction mixture contained 0.2 M-sodium acetate buffer, pH 4.0 or pH 5.5, cerebroside 3-sulphate (56 nmol, 71 322 c.p.m.) [water suspension of cerebroside 3-sulphate prepared as described by Mehl & Jatzkewitz (1963)] and 0.8 unit (91 µg) of enzyme protein in a total volume of 0.2 ml. (The enzyme unit is defined in Table 1.) The tubes were incubated for 1 h at 37°C and the reaction was terminated by heating in a boiling-water bath for 30 s. Immediately after being heated the mixture was cooled in ice, and precipitated protein was removed by centrifugation. A sample of the supernatant was streaked in a 1 cm band on Whatman 3MM paper. Descending chromatography was done in isobutyric acid–0.5 M-ammonia (5/3, v/v) (Suzuki & Strominger, 1960). Areas corresponding to [³⁵S]sulphate were cut out from the paper and radioactivity was measured in a Packard Tri-Carb liquid-scintillation counter as described by George & Bachhawat (1970). In the control reaction mixture the enzyme was added at the end of the incubation period.

pH	Components	Sulphate released (c.p.m.)
4.0	Complete system	41 300 (32 nmol)
	Complete system with boiled enzyme	1024 (0.8 nmol)
5.5	Complete system	11 700 (9 nmol)
	Complete system with boiled enzyme	602 (0.47 nmol)

concentrated by Aquacide I treatment and dialysed as described above. This eluted enzyme also had cerebroside 3-sulphate-degrading activity.

Discussion

The behaviour of the purified chicken brain arylsulphatase, especially the K_m value, anomalous time-activity relationship and inhibitory effect of sulphite, phosphate and sulphate ions suggested that it might be classified as an arylsulphatase A. Like human liver arylsulphatase A (Baum *et al.*, 1958) two pH optima were obtained at different times of incubation, i.e. pH 5.5 for 5–20 min and pH 5.0 for 30–90 min. Balasubramanian & Bachhawat (1963) found only one pH optimum, 4.5, for 10 min incubation for human brain arylsulphatase A. Harinath & Robins (1971) also reported only one pH optimum, 5.7, at different times of incubation for human brain arylsulphatase A with 4-methylumbelliferone sulphate as substrate. Further, the chicken brain arylsulphatase showed an anomalous time-activity relationship (Baum & Dodgson, 1958; Farooqui & Bachhawat,

1971) and the shape of time-activity curve markedly changed in the presence of pyrophosphate ions, as reported by Baum & Dodgson (1958). Harinath & Robins (1971) found, with 4-methylumbelliferone sulphate as substrate, that the human brain arylsulphatase A did not show any anomalous time-activity relationship. These differences in the kinetic properties may be attributed to the different type of substrate employed for the kinetic studies. Except for Cu^{2+} , other metal ions employed in the present study had no inhibitory effect on the enzyme activity. Moreover Ag^+ , which was reported to be inhibitory for ox and human brain arylsulphatase A (Bleszynski & Leznicki, 1967; Harinath & Robins, 1971), was found to have a stimulatory effect on chicken brain arylsulphatase A.

The inhibition of chicken and human brain arylsulphatase A by carbonyl reagents in the presence of traces of Cu^{2+} was in agreement with Roy's (1970) work. The actual mechanism of inhibition is not known. However, Roy (1970) had suggested two possible mechanisms for this inhibition. In the first mechanism he proposed that the enzyme reacted with Cu^{2+} and the carbonyl reagent to give a ternary complex, which was enzymically inactive. In the second possibility Cu^{2+} was reduced to Cu^+ by the carbonyl reagent and the latter was the actual inhibitor of enzyme activity. Ascorbate alone had no effect on enzyme activity in our system, but it enhances the inhibition by Cu^{2+} . The second mechanism of inhibition as postulated by Roy (1970) therefore seems to be more probable.

Chicken brain arylsulphatase A, however, resembled arylsulphatase B of other animal species in its electrophoretic mobility, binding with zinc acetate (Farooqui & Bachhawat, 1971) and the stimulation of enzyme activity by silver nitrate. Roy (1963) reported that livers of marsupials and lower mammals, which were more closely related to birds during evolution, contain a single type II arylsulphatase with properties intermediate between those of arylsulphatases A and B of ox liver. Red-kangaroo liver arylsulphatase A, which was purified by Roy (1971), showed less pronounced anomalous kinetics, did not polymerize at low pH values, had a different isoelectric point and different K_i for sulphate ion compared with arylsulphatase A of ox liver. Thus, the chicken brain arylsulphatase did not correspond to either arylsulphatase A or arylsulphatase B but rather had combined properties of both.

The role of brain arylsulphatases is obscure. The first suggestion about the role of arylsulphatase A came from the work of Austin *et al.* (1963) who reported that arylsulphatase activity was very low in a metachromatic-leucodystrophy patient compared with normal subjects. Austin *et al.* (1964) demonstrated the deficiency of arylsulphatase A not only in the tissue but also in urine of metachromatic-leuco-

dystrophy patients. Further, arylsulphatase A was shown to be a component of cerebroside sulphatase, an enzyme isolated from pig kidney cortex, which could degrade cerebroside 3-sulphate in the presence of a heat-stable polymer (Mehl & Jatzkewitz, 1968).

In the present study it was observed that the purified chicken brain arylsulphatase A had cerebroside 3-sulphate-degrading activity in the absence of a heat-stable polymer at pH 4.0 and 5.5. This degradation in the absence of a heat-stable polymer is particularly notable because this enzyme had certain different properties compared with arylsulphatase A of other animal species. Because the enzyme was heterogeneous, a definite conclusion regarding the role of a heat-stable polymer for the degradation of cerebroside 3-sulphate cannot be made. However, even after the electrophoresis, the protein band corresponding to arylsulphatase A, was able to degrade cerebroside 3-sulphate.

We thank Dr. A. S. Balasubramanian for helpful discussions. The work was supported by grant no. NIH-01-024-1 from the National Institutes of Health, U.S. Public Health Service. A.A.F. is a junior research fellow of the Council of Scientific and Industrial Research, India.

References

- Allen, E. & Roy, A. B. (1968) *Biochim. Biophys. Acta* **168**, 243
- Austin, J. H., Balasubramanian, A. S., Pattabiraman, T. N., Saraswathi, S., Basu, D. K. & Bachhawat, B. K. (1963) *J. Neurochem.* **10**, 805
- Austin, J., McAfee, D., Armstrong, D., O'Rourke, M., Shearer, L. & Bachhawat, B. K. (1964) *Biochem. J.* **93**, 15c
- Balasubramanian, A. S. & Bachhawat, B. K. (1962) *Biochim. Biophys. Acta* **59**, 389
- Balasubramanian, A. S. & Bachhawat, B. K. (1963) *J. Neurochem.* **10**, 201
- Baum, H. & Dodgson, K. S. (1958) *Biochem. J.* **69**, 573
- Baum, H., Dodgson, K. S. & Spencer, B. (1958) *Biochem. J.* **69**, 567
- Bleszynski, W. & Leznicki, A. (1967) *Enzymologia* **33**, 373
- Bleszynski, W., Leznicki, A. & Lewosz, J. (1969) *Enzymologia* **37**, 314
- Chrambach, A., Reisfeld, R. A., Wyckoff, M. & Zaccari, J. (1967) *Anal. Biochem.* **20**, 150
- Farooqui, A. A. & Bachhawat, B. K. (1971) *J. Neurochem.* **18**, 635
- Fiske, C. H. & Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375
- Folch, J., Lees, M. J. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497
- Gatt, S. & Rapport, M. M. (1966) *Biochim. Biophys. Acta* **113**, 567
- George, E. & Bachhawat, B. K. (1970) *Clin. Chim. Acta* **30**, 317
- Harinath, B. C. & Robins, E. (1971) *J. Neurochem.* **18**, 245
- Jerfy, A. & Roy, A. B. (1969) *Biochim. Biophys. Acta* **175**, 355
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265
- Mehl, E. & Jatzkewitz, H. (1963) *Hoppe-Seyler's Z. Physiol. Chem.* **331**, 292
- Mehl, E. & Jatzkewitz, H. (1968) *Biochim. Biophys. Acta* **151**, 619
- Nichol, L. W. & Roy, A. B. (1964) *J. Biochem. (Tokyo)* **55**, 643
- Plapp, B. V. & Cole, R. D. (1966) *Arch. Biochem. Biophys.* **116**, 193
- Rouser, G., Bauman, A. J., Kristchevsky, G., Heller, D. & O'Brien, J. S. (1961) *J. Amer. Oil Chem. Soc.* **38**, 544
- Rouser, G., Bauman, A. J. & Kristchevsky, G. (1967) *Lipid Chromatogr. Anal.* **1**, 137
- Roy, A. B. (1963) *Aust. J. Exp. Biol. Med. Sci.* **41**, 331
- Roy, A. B. (1970) *Biochim. Biophys. Acta* **198**, 76
- Roy, A. B. (1971) *Biochim. Biophys. Acta* **227**, 129
- Sussman, H. H. & Laga, E. (1968) *Biochim. Biophys. Acta* **151**, 281
- Suzuki, S. & Strominger, J. L. (1960) *J. Biol. Chem.* **235**, 257
- Szulzycka, J. G. & Komoszynski, M. (1970) *Acta Biochim. Pol.* **17**, 185