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PURIFICATION, PROPERTIES AND GLYCOPROTEIN NATURE OF ARYLSULFATASE A FROM SHEEP BRAIN

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Summary

A simple and rapid method for the purification of arylsulfatase A (EC 3.1.6.1) from sheep brain has been developed. This includes the concanavalin A-Sepharose affinity chromatography and the pH-dependent polymerization and depolymerization of the enzyme. By these methods a homogeneous enzyme was obtained and the enzyme was purified 7180-fold. Sheep brain arylsulfatase A has been shown to be a glycoprotein containing 25% neutral sugar and 0.5% sialic acid. The constituent neutral sugars were identified as glucose and mannose.

Introduction

The importance of arylsulfatase A was recognized when it was demonstrated that this enzyme is involved in the metabolism of cerebroside-3-sulfate, one of the important constituents of myelin sheath [1,2]. Although considerable information regarding the enzyme and its glycoprotein nature is available from liver [3,4] the glycoprotein nature of arylsulfatase A in the tissue in which it is most affected in metachromatic leukodystrophy namely brain is not known. Arylsulfatase A has been purified from brain by various workers but very little kinetic and other information regarding the enzyme is available [5–8]. From earlier studies it was also observed that although arylsulfatase A is absent in certain form of metachromatic leukodystrophy, considerable amount of arylsulfatase B was present in this disease indicating that these two proteins may have different genetic origin. Goldstone et al. [9] earlier showed that kidney arylsulfatase A could be converted to B form by removal of sialic acid. But later Graham and Roy [4] clearly showed that by neuraminidase treatment purified ox liver arylsulfatase A could not be converted to B form and there was no change in the physical or chemical properties of the enzyme.

Recently Goldstone et al. [10] have also suggested that by neuraminidase treatment, arylsulfatase A to B conversion may not be occurring and only a change from acidic to basic form of the enzyme takes place. Since the glycoprotein nature of arylsulfatase A has not been established in brain it was of interest to purify and study its glycoprotein nature and compare it with that of ox liver arylsulfatase A.

It has been shown earlier from our laboratory that lysosomal glycoprotein enzymes bind with concanavalin A and could be dissociated by the specific sugar and this method could be utilized for the purification of glycoprotein enzymes [11,12].

The present paper describes a simple and rapid method for the purification of arylsulfatase A from sheep brain. This involves the affinity chromatography on concanavalin A-Sepharose and the pH-dependent polymerization and depolymerization of the enzyme. The glycoprotein nature of the enzyme is established and it has been shown that neuraminidase treatment did not convert arylsulfatase A to B form.

Materials and Methods

Sephadex G-200, DEAE-Sephadex A-50, Sepharose 6B, Blue dextran were purchased from Pharmacia, Uppsala, Sweden. γ -globulin, crystalline bovine serum albumin, ovomucoid trypsin inhibitor, cytochrome *c*, Coomassie brilliant blue were obtained from Sigma Chemical Co., U.S.A. Acrylamide and *N,N*-methylene bisacrylamide were obtained from Eastman Organic Chemicals, U.S.A. *Vibrio cholerae* neuraminidase was obtained from Behringwerke AG, Germany. All other chemicals used were of analytical grade.

Methods. The nitrocatechol sulfate was prepared according to the method of Dodgson and Spencer [13] and was purified according to the method of Ahmad and Bachhawat (unpublished observation) by passing through Sephadex G-10 and the retarded material which appeared in the inner volume was collected and used as substrate. Concanavalin A was prepared from jack bean according to the method of Surolia et al. [14] and the concanavalin A-Sepharose was prepared as described by Bishayee and Bachhawat [11] except that Sepharose 6B was used instead of Sepharose 4B. This preparation had 16 mg of concanavalin A bound per ml of packed Sepharose 6B as judged by the protein content of the washings.

Protein estimation. Protein was determined by the method of Lowry et al. [15] with crystalline bovine serum albumin as standard.

Carbohydrate analysis of enzyme. Neutral sugar content of the enzyme was estimated by phenol-sulphuric acid method of Roughan and Batt [16] using galactose as standard. For identification of neutral sugars the enzyme (250 μ g protein) was hydrolysed at 100°C with 1M HCl for 5 h in a sealed tube and processed according to the method of Spiro [17]. The sugars were identified by paper chromatography using the solvent system of Mukherjee and Sri Ram [18]. After drying the paper the reducing sugar spots were identified by staining with alkaline silver nitrate.

During Sephadex column operation for the purification of the enzyme it

was observed that occasionally phenol-sulfuric acid-positive material was leached out from the column and to remove this interfering material the enzyme solution was dialyzed against saturated $(\text{NH}_4)_2\text{SO}_4$ and the precipitated enzyme was dissolved in 0.01 M Tris/acetate buffer, pH 7.4, and dialysed against the same buffer.

Sialic acid determination and neuraminidase treatment. Sialic acid content of the enzyme was determined according to the thiobarbiturate assay of Warren [19] as modified by Saifer and Gerstenfeld [20]. The enzyme was desialylated by incubating 100 μg of purified enzyme with 10 units of *V. cholerae* neuraminidase in 0.1 M Tris/acetate buffer, pH 7.4 for 3 h at 37°C in a total volume of 0.5 ml in presence of 0.06 M NaCl and 4 mM CaCl_2 . The desialylated enzyme was then dialyzed against 0.01 M Tris/acetate buffer, pH 7.4. Neuraminidase was omitted from the control tubes. The sialic acid content of the desialylated enzyme was too low and as such it was not possible to determine the sialic acid content with the available material.

Gel electrophoresis. Polyacrylamide gel electrophoresis with 7.5% gel was carried out by the method of Ornstein and Davis [21] in 0.04 M barbitone buffer, pH 8.6, for 3 h using 3 mA/tube. The gels were stained by Coomassie brilliant blue according to the method of Chrambach et al. [22]. Periodic acid-Schiff staining for glycoprotein was done according to the method of Kapitany and Zebrowski [23]. Sodium dodecylsulfate-polyacrylamide gel electrophoresis for subunit molecular weight determination was carried out according to the method of Weber and Osborn [24], using γ -globulin, crystalline bovine serum albumin, ovalbumin, myoglobin and cytochrome *c* as standard.

Molecular weight determination. Molecular weight determination of the purified enzyme by gel filtration was carried out according to the method of Andrews [25] using Sephadex G-200 equilibrated with 0.02 M Tris/acetate buffer, pH 7.4, containing 0.1 M NaCl. γ -Globulin, bovine serum albumin, ovalbumin, ovomucoid trypsin inhibitor and cytochrome *c* were used to standardise the column.

Binding study of arylsulfatase A to Ricinus communis agglutinin 1-Sepharose column. A study was undertaken to investigate the binding of purified arylsulfatase A to *Ricinus communis* agglutinin 1, a galactose binding protein isolated from *Ricinus communis*. This is a lectin which specifically binds with glycoproteins containing terminal β -galactose [26,27]. Enzyme solution having 12 μg protein was passed through a column of *Ricinus communis* agglutinin 1 covalently attached to Sepharose 4B previously equilibrated with 0.02 M acetate buffer, pH 5.6, containing 0.2 M NaCl. It was observed that there was no binding of arylsulfatase A and all the enzyme appeared in the breakthrough.

Arylsulfatase assay. Total arylsulfatase activity was assayed according to the method of Worwood et al. [28]. During the first two steps of the enzyme purification the combined activities of arylsulfatase A and B present were obtained. The incubation mixture consisted of 0.2 ml of diluted enzyme and 0.2 ml of 0.02 M nitrocatechol sulfate in 0.5 M sodium acetate solution adjusted with acetic acid to pH 5.6. Incubation was done at 37°C for 10 min after which the reaction was stopped by the addition of 2.5 ml of 1 M NaOH and the colour developed was measured in the Klett using 50 filter. Since zinc acetate fractionation partially separates arylsulfatase A and B as observed by Farooqui

and Bachhawat [29], in the latter steps the measure of the enzyme activity according to the above method was mainly due to arylsulfatase A. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of nitrocatechol from *p*-nitrocatechol sulfate in 1 min under the assay conditions. The specific activity was expressed as units/mg protein.

Purification of arylsulfatase A. The first three steps of the purification, namely preparation of crude homogenate, alcohol extraction and zinc acetate fractionation were carried out as described by Farooqui and Bachhawat [29] except that this was done at 0 instead of -5°C .

(a) *Concanavalin A-Sepharose affinity chromatography:* Zinc acetate supernatant which contained mainly arylsulfatase A was kept at room temperature overnight which resulted in precipitation of some of the proteins. This was centrifuged at $12\,000 \times g$ for 30 min and a small aliquot was dialysed against 0.001 M Tris/acetate buffer, pH 7.4, to assay the enzyme. Rest of the supernatant (905 ml) containing 1.64 g protein was passed through a concanavalin A-Sepharose column (3.5×2.3 cm) at a rate of 60 ml/h at room temperature (28°C) previously equilibrated with 0.02 M Tris/acetate buffer, pH 7.4. After passing the supernatant the column was washed with five bed volumes of the same buffer and then eluted with a solution containing 10% α -methyl-D-glucoside in the same buffer. The enzyme was eluted as a sharp peak as shown in Fig. 1. The active fractions were pooled and a small portion was dialysed against 0.001 M acetate buffer, pH 5.0.

(b) *DEAE-Sephadex A-50 column chromatography:* The eluate from concanavalin A-Sepharose column containing 8.7 mg protein was then passed through a DEAE-Sephadex A-50 column (6×1 cm) at a rate of 15 ml/h

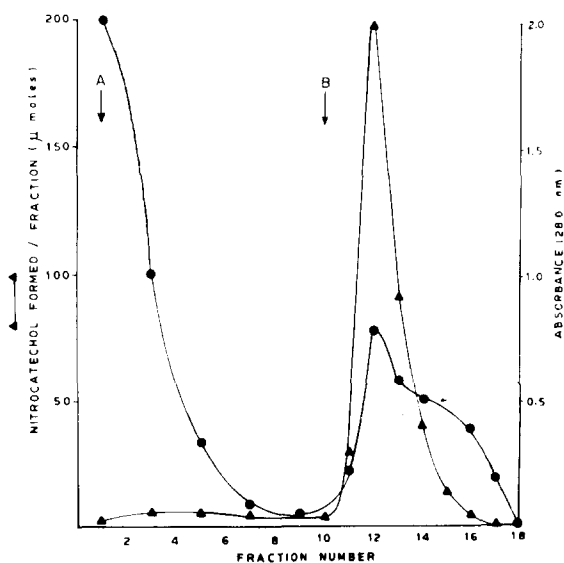


Fig. 1. Elution profile of arylsulfatase A from concanavalin A-Sepharose column. 5-ml fractions were collected and analysed for enzyme activity as described in Methods. (A) Washing the column with 0.02 M Tris/acetate buffer, pH 7.5. (B) Elution with 0.02 M Tris/acetate buffer, pH 7.5, containing 10% methyl glucoside. ●, absorbance at 280 nm; ▲, arylsulfatase activity.

previously equilibrated with 0.02 M Tris/acetate buffer, pH 7.4. The column was first washed with the same buffer and then with 0.05 M acetate buffer, pH 5. The enzyme was eluted with 0.1 M acetate buffer, pH 5, containing 0.1 M NaCl. The active fractions were pooled and dialysed against 0.001 M acetate buffer, pH 5, whereby the enzyme was precipitated. This was centrifuged at $12\,000 \times g$ for 30 min and the precipitate was dissolved in 0.1 M acetate buffer, pH 5. This contained approx. 1 mg protein in 2 ml.

(c) *Gel filtration on Sephadex G-200 at pH 5*: This enzyme solution was placed on a 65×1.5 cm column of Sephadex G-200 equilibrated with acetate buffer, pH 5, $I = 0.12$ (final concentrations were 0.05 M NaCl, 0.07 M sodium acetate and 0.03 M acetic acid), as described by Woorwood et al. [28]. Flow rate was maintained at 15 ml/h and 5-ml fractions were collected. The enzyme appeared in the void volume and the active fractions were pooled, dialysed against 0.01 M Tris/acetate buffer, pH 7.4, and concentrated. This contained 0.4 mg protein in 2 ml.

(d) *Gel filtration on Sephadex G-200 at pH 7.5*: This enzyme was then placed on a 65×1.5 cm column of Sephadex G-200 equilibrated with barbiturate buffer, pH 7.5, $I = 0.1$ (final concentrations were 0.09 M NaCl, 0.01 M sodium diethyl barbiturate, 0.02 M barbituric acid). In this condition the enzyme assumed its lower molecular weight form and appeared in the inner volume. The active fractions were pooled, dialysed against 0.001 M Tris/acetate, pH 7.4, and concentrated. This contained 0.3 mg protein in 1.6 ml.

Results and Discussion

Sheep brain arylsulfatase A was purified 7180-fold starting from the homogenate with a recovery of 21.5% starting from zinc acetate supernatant (Table I). Since the homogenate contain the mixture of the two enzymes A and B and the method of assay does not differentiate these two enzymes, the recovery was calculated from the zinc acetate supernatant. The preparation was homogeneous on acrylamide gel electrophoresis. The methods involved are zinc

TABLE I
PURIFICATION OF ARYLSULFATASE A FROM SHEEP BRAIN

Purification steps	Total volume (ml)	Total protein (g)	Total enzyme units	Specific activity	Fold purification	Yield* (%)
Homogenate	1470	38.22	352	0.009	—	—
Alcohol supernatant	1080	7.8	194	0.025	2.8	—
Zinc acetate supernatant	965	1.84	88	0.054	6.0	100
Concanavalin A-Sepharose affinity chromatography	26	0.0087	52	5.4	600	59
DEAE-Sephadex chromatography	30	0.0028	31	10.5	1170	35
Low ionic strength precipitation	2	0.001	29	31.0	3444	33
Sephadex G-200 gel filtration at pH 5	2	0.0004	23	59.0	6555	26
Sephadex G-200 gel filtration at pH 7.5	1.6	0.0003	19	64.6	7180	21.5

* The activity in zinc acetate supernatant was arbitrarily taken as the initial concentration of arylsulfatase A as discussed in the text.

acetate fractionation, concanavalin A-Sepharose affinity chromatography, low ionic strength pH 5 precipitation and gel filtration under the conditions where the enzyme is polymerized and depolymerized at pH 5 and 7, respectively. The yield of the material was 0.5–0.6 mg protein per kg of brain which closely agree with the reported yield for ox liver arylsulfatase A [3].

Earlier work from this laboratory has shown that brain arylsulfatase A binds with concanavalin A-Sepharose and could be eluted with α -methyl-D-glucoside. This suggests that the brain sulfatase A is a glycoprotein. In the present study using affinity chromatography a 100-fold purification was achieved in a single step with 50% recovery. It is known that concanavalin A-glycoprotein interaction is temperature dependent [30] and as such column operation was carried out at room temperature (28°C).

Gel electrophoresis

Fig. 2 shows the electrophoretic pattern of the enzyme from various stages of purification. It can be seen that the final preparation was homogeneous. Periodic acid-Schiff staining for glycoprotein as well as the enzyme staining were done and these bands coincided with the protein band. Native

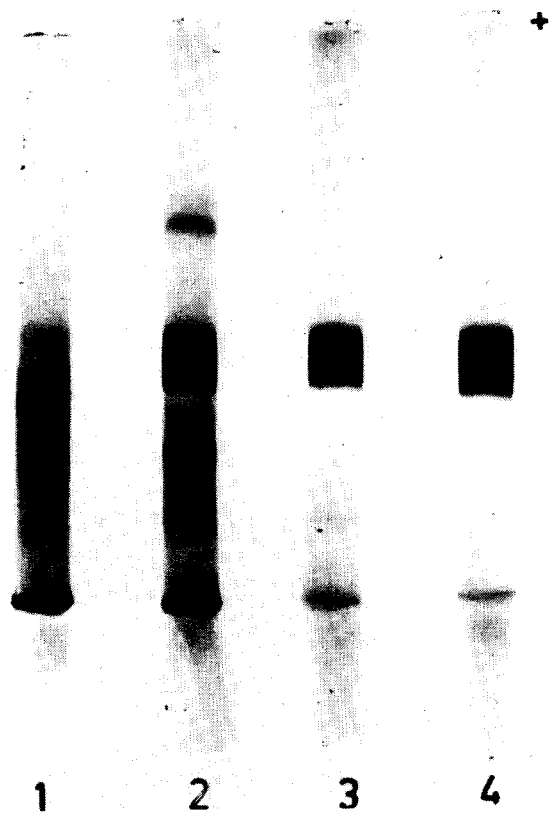


Fig. 2. Gel electrophoresis pattern of arylsulfatase A from various stages of purification. (1) Eluate from concanavalin A-Sepharose column. (2) Eluate from DEAE-Sephadex column. (3) Enzyme after gel filtration on Sephadex G-200 at pH 5. (4) Enzyme after gel filtration on Sephadex G-200 at pH 7.5.

and desialylated enzyme had the same electrophoretic mobility of 2.3 cm whereas Graham and Roy [4] had observed slight difference in the mobility of the native and desialylated enzyme.

Molecular weight

Molecular weight of 122 000 was obtained for the purified arylsulfatase A at pH 7.5 using the gel filtration method and this represents the molecular weight of the monomeric enzyme. This enzyme also showed an ability to polymerize at pH 5. Nichol and Roy [31] observed a molecular weight of 107 000 for the monomer and 411 000 for the tetramer of ox liver arylsulfatase A. Similarly Worwood et al. [28] observed a molecular weight of 130 000 at pH 7.5 and 400 000 at pH 5, for rat liver arylsulfatase A.

The subunit molecular weight was determined using sodium dodecylsulfate electrophoresis which gave a value of 63 000. Considering the molecular weight of the monomer enzyme as 122 000, it can be assumed that sheep brain arylsulfatase A is composed of two identical subunits. Since the polysaccharide portion of the enzyme does not bind to sodium dodecylsulfate, and the enzyme contain higher amount of sugar (25%) the estimated molecular weight by this method may not be the correct one. Roy and Jerfy [32] observed that ox liver arylsulfatase A on sodium dodecylsulfate treatment dissociated into half units of molecular weight of 55 000. Similarly in 6 mM dithiothreitol and 8 M urea dissociation to material of molecular weight of 55 000 was complete and small amounts of material of molecular weight 27 000 were also produced. It was suggested by these workers that fundamental subunit of sulfatase A has a molecular weight of 27 000 and that four of these make up the native enzyme.

The carbohydrate content of arylsulfatase A

Two independent estimations of the carbohydrate content of sulfatase A gave the following analysis: neutral sugar, 24.7 and 25.2%; sialic acid, 0.49 and 0.37%. Earlier Graham and Roy [4] showed that ox liver arylsulfatase A contained 3.85% neutral sugar and 2.18% sialic acid. Brain enzyme seems to contain higher amounts of neutral sugar as compared to liver enzyme. It may be mentioned that other brain enzymes like alkaline phosphatase [33] and β -N-acetyl hexosaminidase [34] have been shown to contain higher amounts of neutral sugar namely 40 and 27%, respectively. Identification of neutral sugars by paper chromatography as shown in Fig. 3 suggested that glucose and mannose are present in sheep brain arylsulfatase A, glucose being in greater amounts. It is interesting to note that even the nature of neutral sugar present in sheep brain arylsulfatase A is quite different from that of ox liver arylsulfatase A. Graham and Roy [4] observed that ox liver enzyme contains galactose and mannose predominantly and traces of glucose and fucose were also present whereas sheep brain enzyme was devoid of any galactose. The absence of terminal galactose was supported by the low sialic acid content and also by its inability to bind with *R. communis* agglutinin 1-lectin. The presence of glucose in the enzyme supports the observation made by Van Nieuw Amerongen et al. [35] on the brain glycoprotein who observed the presence of 4% glucose in their preparation. Moreover purified alkaline phosphatase [33] and β -N-acetyl hexosaminidase [34] from brain were shown to contain glucose as

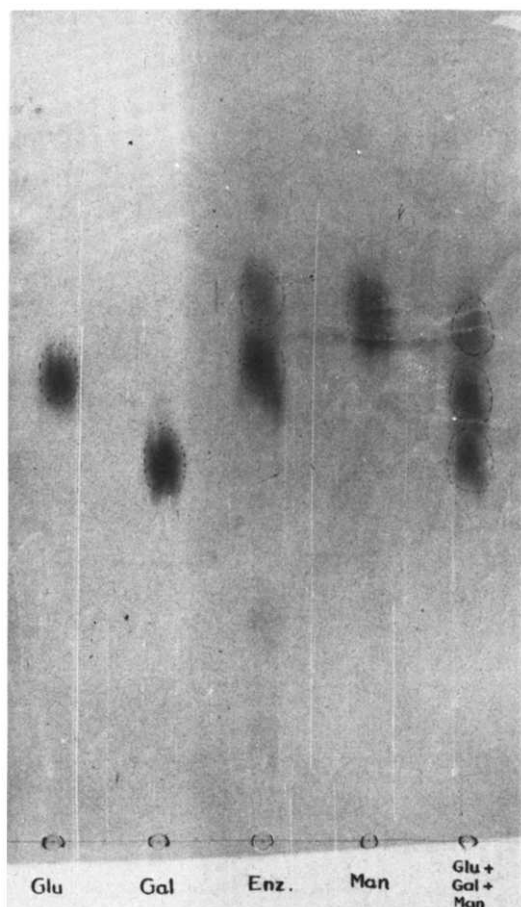


Fig. 3. Paper chromatographic pattern of neutral sugars of arylsulfatase A. The conditions of the experiment were as described in the text. Enz indicates arylsulfatase A. Glucose, mannose, galactose and the mixture are indicated by Glu, Man, Gal and Glu + Gal + Man, respectively.

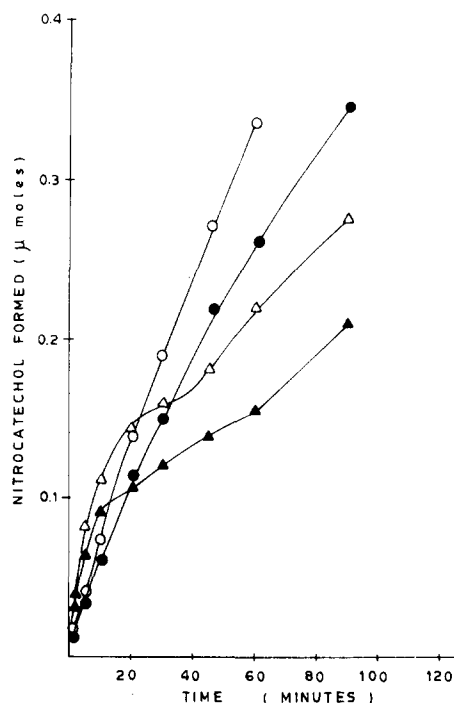


Fig. 4. Time vs activity curve of native and desialylated arylsulfatase A at two different temperatures. The incubation mixture consisted of 0.25 M acetate buffer, pH 5.6, 5 mM nitrocatechol sulfate, 0.01% bovine serum albumin and 0.2 μ g of native enzyme or 0.18 μ g of desialylated enzyme in a total volume of 0.4 ml. The reaction mixture was incubated at 37 or 15°C, for various time intervals and the reaction was stopped as indicated in Methods. ▲, desialylated enzyme at 37°C; ●, desialylated enzyme at 15°C; △, native enzyme at 37°C; ○, native enzyme at 15°C.

one of the constituent neutral sugars. It may be mentioned here that this is the first report on the glycoprotein nature of brain arylsulfatase A. This suggests that not only the total neutral sugar content but also the type of sugar in glycoprotein varies from tissue to tissue.

Kinetic properties of arylsulfatase A

K_m determined with Nitrocatechol sulfate and *p*-nitrophenyl sulfate were found to be 0.6 and 45 mM, respectively. pH optimum of 5.6 was obtained which was comparable with other reported values [3,7,28]. Sulfate, sulfite, phosphate and pyrophosphate were found to be competitive inhibitors and the

K_i values of $0.35 \cdot 10^{-2}$, $0.7 \cdot 10^{-5}$, $0.18 \cdot 10^{-4}$ and $0.4 \cdot 10^{-4}$ M. respectively, were obtained. It was observed that there was no difference in the kinetic properties of the native and desialylated enzyme (Fig. 4). The enzyme had a turnover number of 16 100. These kinetic data were almost similar to that reported for ox liver enzyme [3]. However, in some of the kinetic properties the values reported here were different from those reported earlier from this laboratory using crude sheep brain enzyme and crude substrate [29]. This discrepancy is not clear at present but it may be due to the impure enzyme and impure substrate used in earlier experiments.

Acknowledgements

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