Biochimica et Biophysica Acta, 616 (1980) 218—227

BBA 69152

A VARIANT FORM OF ARYLSULFATASE A IN HUMAN URINE DERIVED DIRECTLY FROM THE RENAL PELVIS

KINETIC AND IMMUNOLOGICAL CHARACTERIZATION

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(Received August 12th, 1980)

Key words: Arylsulfatase A variant; Kinetics; (Human urine)

Summary

Arylsulfatase A (aryl-sulfate sulfohydrolase, EC 3.1.6.1) was examined in voided and in nephrostomic urine. A variant form of the enzyme was found in nephrostomic urine, in addition to the minor form, which is the sole component of arylsulfatase A in voided urine. The nephrostomic enzyme differed from the voided urine enzyme with respect to the kinetic parameters, the isoelectric point, heat stability and immunological reactivity. The isoelectric points of the voided urine and nephrostomic enzymes were 4.7 and 5.3, respectively. The nephrostomic enzyme was more heat-labile at 62.5°C than the voided urine enzyme. Although the K_m values of the two enzymes with nitrocatechol sulfate as substrate were almost the same, the V value of the nephrostomic enzyme was approx. one-hundredth that of the voided urine enzyme. The molecular weight (almost 130 000) did not differ between the voided urine and nephrostomic enzymes. It was demonstrated by various methods, using IgG antibody against the purified voided urine enzyme, that the nephrostomic enzyme was antigenically distinct from the voided urine enzyme.

Introduction

Arylsulfatase (arylsulfate sulfohydrolase, EC 3.1.6.1) catalyzes the hydrolysis of the ester bond in various aromatic sulfates [1]. Three arylsulfatases are known: two of them, A and B, are lysosomal enzymes which are easily extracted; the third one, C, is insoluble and localized in the microsomes [1].

The first two arylsulfatases have a greater affinity for nitrocatechol sulfate than *p*-nitrophenyl sulfate, while the third one, C, has a greater affinity for the latter substrate [1].

The physiological role of arylsulfatase A has already been established [2]. It has been reported that arylsulfatase A is a cerebroside 3-sulfatase and that its deficiency causes the disease of metachromatic leucodystrophy, which is characterized by an abnormal amount of cerebroside 3-sulfate in various organs and by excessive excretion of this lipid in the urine [2,3]. The defects of this enzyme have been observed also in the urine of affected patients [2].

Arylsulfatase activity in human urine was first noticed by Huggins and Smith [4]. Boyland et al. [5] observed the increased activity of arylsulfatase in several infected urines and in urines from patients with carcinoma of the bladder and other sites. Arylsulfatase A has been isolated from various human organs, including the brain [6], liver [7] and kidney [8]. The enzyme has also been isolated from urine and investigated in detail [9,10], however, these studies have been performed only with voided urine

This paper reports that arylsulfatase A in human urine of the nephrostomic tube is kinetically and antigenically different from the enzymes of voided urine.

Materials and Methods

Chemicals. The following chemicals were obtained from commercial sources: Nitrocatechol sulfate, *p*-nitrophenyl sulfate and *p*-acetylphenyl sulfate (Sigma Chemical Co.); L-tyrosine-O-sulfate (donated by Dr. S. Gasa, Hokkaido University); DEAE-cellulose (Type DE-52, Whatman Co.); Sephadex G-200 and Con A-Sepharose (Pharmacia Fine Chemicals Inc.); Ampholine (LKB Instruments Inc.).

Collection and treatment of urine. Human urine was taken from either the nephrostomic tube (nephrostomic urine) of patients with congenital stenosis of the pelvi-ureteric junction or from spontaneously voided urine (voided urine) in the same individual. Because the urine from the nephrostomic tube may be accompanied by infection, the urine from the cystostomic tube (cystostomic urine) was also examined.

All of the following operations were carried out at 4°C unless otherwise stated. The urine samples were centrifuged to remove cellular debris and treated with a saturated $(NH_4)_2SO_4$ solution to a final 50%. The precipitate was then dissolved in 20 mM Tris-HCl buffer (pH 7.5) and dialyzed extensively against the same buffer. After removal of the insoluble materials by centrifugation, the supernatant was used as an enzyme source. The protein concentrations of the voided urine, nephrostomic and cystostomic enzymes thus obtained were 1.5, 10.5 and 3.3 mg/ml, respectively; however, the enzyme activities were similar in all the samples and the specific activities were 13, 1.9 and 6.1 munits/mg, respectively.

The protein amounts were determined by the method of Lowry et al. [11] using bovine serum albumin as the standard.

Assays for enzyme activity. The enzymes were assayed by measuring the release of nitrocatechol from nitrocatechol sulfate using the procedure of Baum

et al. [12], with a minor modification. The reaction mixture consisted of 0.2 ml sodium acetate buffer (pH 5.0) containing 10 mM nitrocatechol sulfate, 0.5 mM $Na_4P_2O_4$, 10% NaCl and the urine enzyme in a final volume of 0.4 ml. The tubes were incubated at 37°C for 30 min by shaking and the reaction was stopped by the addition of 0.6 ml 1 M NaOH.

The amount of liberated nitrocatechol ($\epsilon_{510nm} = 1.24 \cdot 10^4\ M^{-1} \cdot cm^{-1}$) was determined spectrophotometrically. 1 unit of enzyme activity was defined as 1 μmol nitrocatechol liberated/min under the above conditions.

When other substrates were used the following methods were followed: For *p*-nitrophenyl sulfate, Stumpf [13]; for *p*-acetylphenyl sulfate, Dodgson et al. [14] and for L-tyrosine-O-sulfate, Fluharty et al. [15].

Isoelectric focusing. The samples of urine enzymes prepared as described above were subjected to isoelectric focusing in a column (110 ml) containing 1% Ampholine carrier ampholyte solution, over a pH range of 4—6, with a stepwise sucrose gradient at 500 V for 50 h, according to the method of Vesterberg [16]. The column content was collected separately in 2 ml fractions each at a flow rate of 80 ml/h. The pH of the fraction was measured in an ice-cold bath.

Purification of arylsulfatase A from normal voided human urine. The purification steps are outlined in Table I. 20 l urine were subjected to $(NH_4)_2SO_4$ precipitation (50% saturation). The precipitate was dissolved in 20 mM Tris-HCl buffer (pH 7.5) and dialyzed extensively against the same buffer.

Then the dialysate was applied to the DEAE-cellulose column (Cl^- form) (2.6 × 13 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5). After extensive washing with the equilibration buffer, the enzyme was eluted with a linear gradient of NaCl (0—0.6 M) in the same buffer (250 ml each in a mixing chamber and reservoir).

The pooled fraction of the enzyme was applied to the Con A-Sepharose column (2 × 7 cm) equilibrated with 1 mM $MnCl_2$/1 mM $CaCl_2$/0.5 M NaCl/20 mM Tris-HCl buffer (pH 7.5). After the column was washed with the equilibration buffer until absorbancy of the eluate at 280 nm was nearly zero, the enzyme was eluted with equilibration buffer containing 10% α-methyl-glucoside.

The pooled fraction of the enzyme was concentrated, applied to the

TABLE I

PURIFICATION OF ARYLSULFATASE A FROM NORMAL VOIDED HUMAN URINE

Starting material was 20 l urine. Units are given as μmol nitrocatechol liberated/min.

Procedure	Protein (mg)	Specific activity (units/mg)	Total activity (units)	Yield (%)	Purification (-fold)
Urine	2650	0.005	13.3	100	1
$(NH_4)_2SO_4$ (0—50%)	630	0.02	12.6	94.7	4
DEAE-cellulose	11	0.33	3.6	27.1	66
Con A-Sepharose	1.74	1.16	2.0	15.0	232
Sephadex-G 200	0.75	2.31	1.7	12.8	462

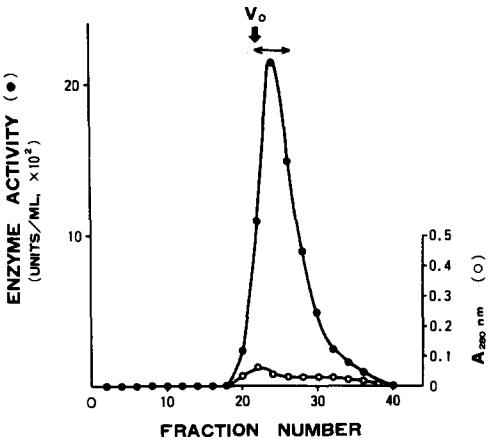

Fig. 1. Sephadex G-200 column chromatography. Fractions of 3.2 ml were collected at a flow rate of 20 ml/h. V_0 refers to void volume. See text for the detailed procedure.

Sephadex G-200 column (0.9 × 37.5 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5) and finally eluted with the same buffer (Fig. 1).

In the final step, 0.75 mg of the purified enzyme was obtained from 20 l urine with 462-fold purification (Table I). The specific activity of the final enzyme preparation was only 2.3 units/mg, compared with the reported value of 59 units/mg by Stevens et al. [10]. Though the present enzyme was not very

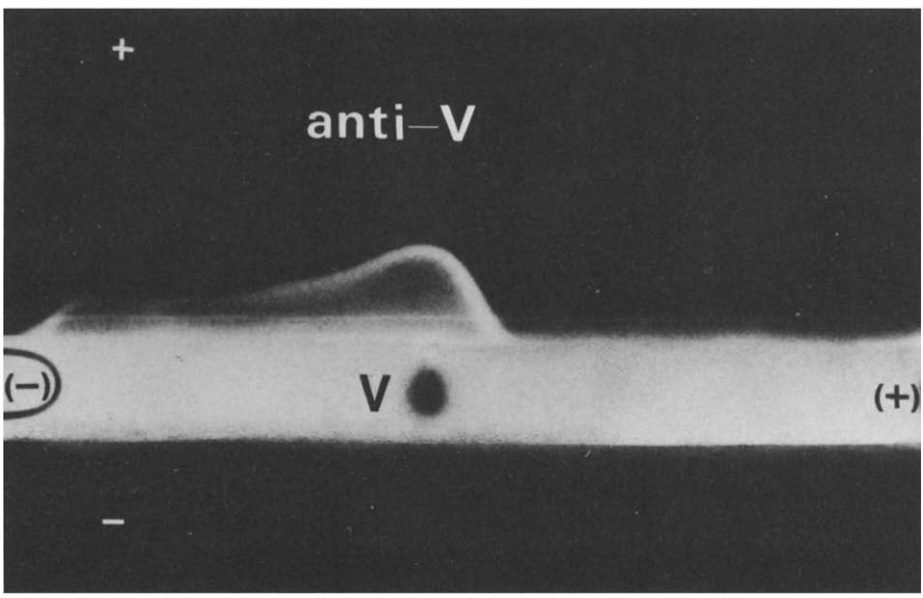

Fig. 2. Crossed immunoelectrophoresis of the purified arylsulfatase A. In the first dimension, the purified voided urine enzyme (20 μg protein) was electrophoresed at 2 mA/gel in the 7.5% polyacrylamide gel. In the second dimension, the plate containing IgG raised against voided urine enzyme (anti-V) was electrophoresed at 5 V/cm in the right direction for the first dimension. The protein in the polyacrylamide gel was stained with 0.1% Coomassie brilliang blue. V, arylsulfatase A purified from voided urine.

highly purified the final preparation was nearly homogeneous, judging from its migration as a single protein band coincident with enzyme activity on 7.5% polyacrylamide gel electrophoresis [17]. Homogeneity was also shown by the denaturating condition of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis [18]. A comparable preparation of the nephrostomic enzyme showed the specific activity of 0.34 units/mg.

Additional evidence for the homogeneity of the final preparation was documented in subsequent experiments, on rabbits which were immunized by the purified enzyme.

Preparation of antiserum against the purified voided urine enzyme. For immunization, 1 ml of a mixture containing an equal volume of complete Freund adjuvant and 10 μg purified arylsulfatase A from voided urine was injected into the back of a New Zealand white rabbit weighing 1 kg. Booster injections were performed at approximate 2-week intervals with the same amount of enzyme. The preinoculation serum was used as the control.

The antiserum was purified by $(NH_4)_2SO_4$ precipitation (50% saturation) and DEAE-cellulose column chromatography [19] to yield the γ-globulin fraction. The monospecific antisera for arylsulfatase A which were obtained did not cross-react with the other urine proteins. This immunological specificity of anti-arylsulfatase A was confirmed by crossed immunoelectrophoresis (Fig. 2).

Immunological techniques. The following methods were used: the Ouchterlony method for double immunodiffusion [20]; the method of Mancini et al. for single radial immunodiffusion [21]; the method of Scheidegger for immunoelectrophoresis [22] and the method of Laurell for crossed immunoelectrophoresis [23].

Results

Properties of arylsulfatase A of voided urine and nephrostomic enzymes

Substrate specificity. As shown in Table II, when nitrocatechol sulfate or L-tyrosine-O-sulfate was used as the substrate, the specific activity of the enzyme extracted from the nephrostomic urine was significantly lowered to about one-seventh that of the enzyme extracted from the voided urine. Although the specific activity of the nephrostomic enzyme using *p*-acetylphenyl sulfate was only a little lower than that of the voided urine enzyme, it was about 2-fold higher when the substrate used was *p*-nitrophenyl sulfate.

TABLE II

SUBSTRATE SPECIFICITY

Assay methods for various substrates are given in Materials and Methods. The protein amounts of the voided urine and nephrostomic enzymes in the reaction mixture were 0.15 and 1.05 mg, respectively. Specific activity is expressed as nmol/min per mg on the respective substrate.

Substrates	Voided urine enzyme	Nephrostomic enzyme
Nitrocatechol sulfate	13.0	1.9
p-Nitrophenyl sulfate	0.2	0.4
p-Acetylphenyl sulfate	0.8	0.6
L-Tyrosine-O-sulfate	0.3	0.05

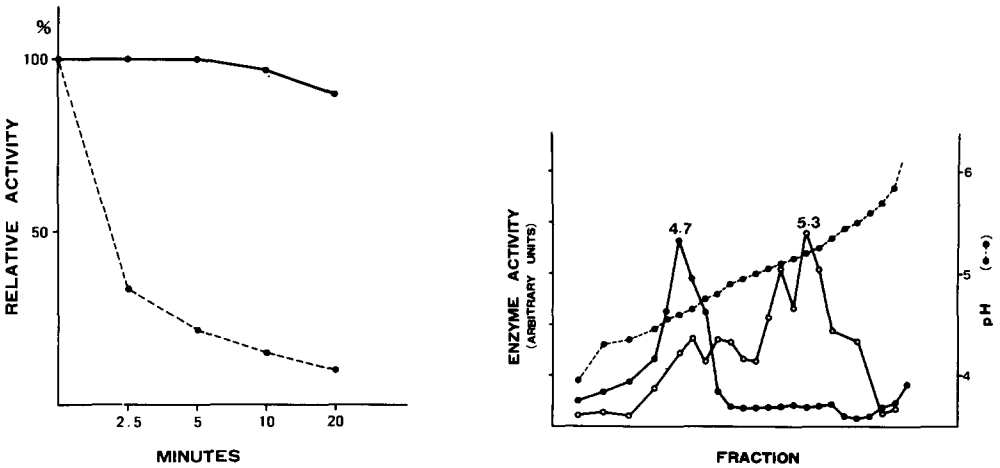

Fig. 3. Heat stability of urine arylsulfatase A. Assay conditions are given in Materials and Methods, except that preincubation at 62.5°C for various intervals was carried out. See text for the detailed procedure. The protein amounts of the voided urine and nephrostomic enzymes in the reaction mixture were 0.15 and 1.05 mg, respectively. ———, voided urine enzyme; - - - - -, nephrostomic enzyme.

Fig. 4. Resolution of arylsulfatase A by isoelectric focusing. The samples of urine enzymes were electrophocused over a pH range of 4—6, as described in Materials and Methods. The protein amounts of voided urine and nephrostomic enzymes applied to the column were 3 and 21 mg, respectively. Voided urine enzyme, (●———●); nephrostomic enzyme (○———○).

The subsequent experiments were performed at the same level of enzyme activity using nitrocatechol sulfate.

Heat stability. Aliquots of the urine enzymes were heated at 62.5°C for various intervals by shaking and frozen immediately in liquid nitrogen. Then the samples were thawed under tap water and assayed for enzyme activity.

The voided urine enzyme was markedly stable even when heat treatment was applied at 62.5°C for 20 min, while the nephrostomic enzyme was more labile and decreased to below one-half of the maximum value when heated at 62.5°C for 2.5 min (Fig. 3).

Kinetic parameters. The optimum pH was near 5.0 for both the voided urine and nephrostomic enzymes. However, the V of the nephrostomic enzyme was approx. one-hundredth of that in the voided urine enzyme (10 and 0.1 units/mg protein, respectively). Upon analytical disc gel electrophoresis at alkaline pH, both the voided urine and nephrostomic enzymes had the same R_F value. Each form of the enzyme was eluted as a single peak in an elution volume corresponding to a molecular weight of near 130 000 on the Sephadex G-200 column.

Effects of various chemicals. As shown in Table III, there was no significant difference in the effects of the chemicals on both the voided urine and nephrostomic enzymes. The enzyme activity was inhibited markedly by PO_4^{3-} in both the voided urine and nephrostomic enzymes.

Resolution by isoelectric focusing. The isoelectric point of the voided urine enzyme was pH 4.7 as a single peak, while the nephrostomic enzyme showed a variety of peaks with a major peak of pH 5.3 (Fig. 4).

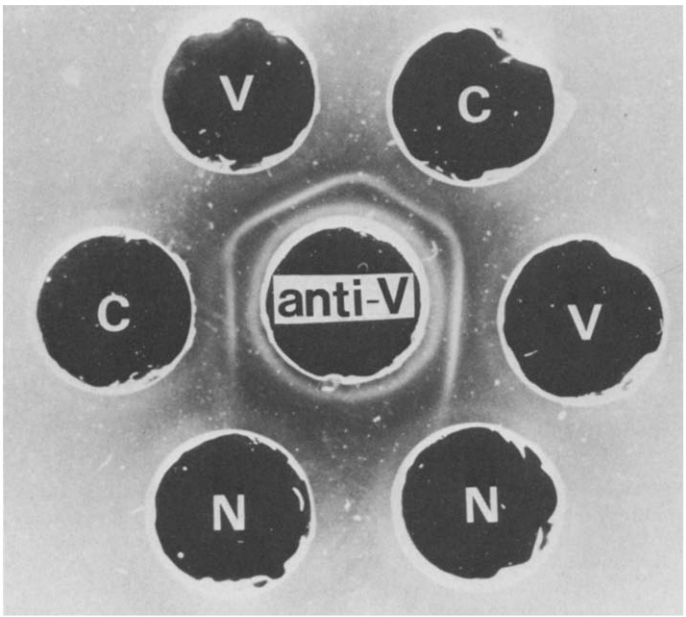

Fig. 5. Double immunodiffusion test between IgG raised against voided urine enzyme (anti-V) and urine enzymes. The voided urine, nephrostomic and cystostomic enzymes were served to double immunodiffusion, and each protein concentration was 10.5 mg/ml.

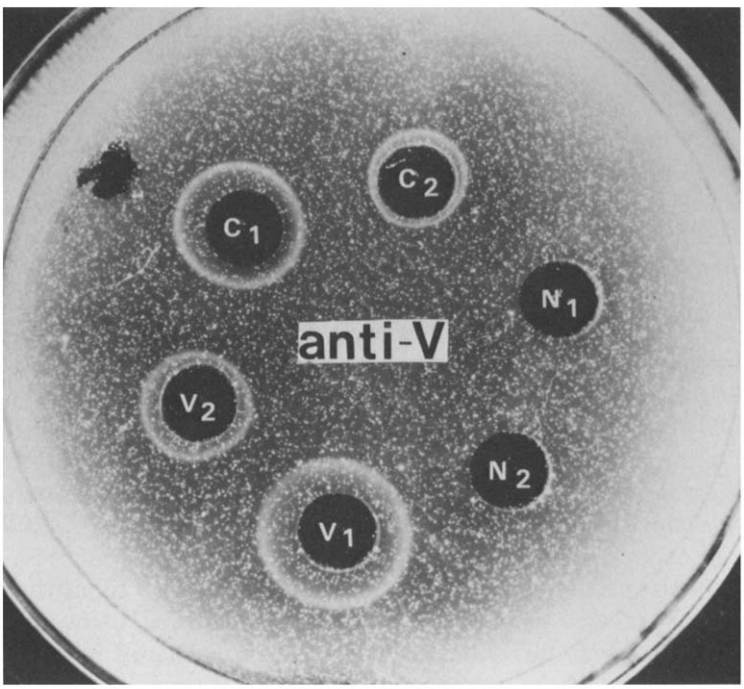

Fig. 6. Single radial immunodiffusion test of arylsulfatase A. The voided urine, nephrostomic and cystostomic enzymes were served to single radial immunodiffusion on a gel plate containing IgG raised against voided urine enzyme (anti-V), and each protein concentration was 10.5 mg/ml. 1, undiluted; 2, 2-fold diluted enzyme.

TABLE III

EFFECTS OF VARIOUS CHEMICALS

Assay procedure is given in Materials and Methods, except that the various chemicals indicated were added. The protein amounts of the voided urine and nephrostomic enzymes in the reaction mixture were 0.15 and 1.05 mg, respectively.

Chemicals	Concentration (mM)	Voided urine enzyme (%)	Nephrostomic enzyme (%)
None		100	100
PCMB *	2.5	63.1	44.5
Na_2SO_4	2.5	81.1	74.8
KCN	2.0	85.1	93.7
EDTA	5.0	98.9	94.1
NaF	2.0	69.9	60.8
Cysteine	2.5	89.2	76.9
Na_3PO_4	2.5	9.7	8.6

* *p*-Chloromercuribenzoate.

Immunochemical properties. Ouchterlony double immunodiffusion studies between IgG raised against voided urine enzyme and different urine enzymes were carried out with solutions diluted serially over a wide range of concentrations. A single precipitin line was seen in the voided urine and cystostomic enzymes, but not in the nephrostomic enzyme (Fig. 5). Since the single precipitin line was seen, the purified antiserum preparation appeared to contain a single protein species which evoked an antibody response.

These immunological specificities were confirmed also by single radial immunodiffusion (Fig. 6) and immunoelectrophoresis (Fig. 7). In the single radial immunodiffusion test, there was direct relationship between the size of the precipitin ring and the dilution of the voided urine and cystostomic enzymes observed (Fig. 6).

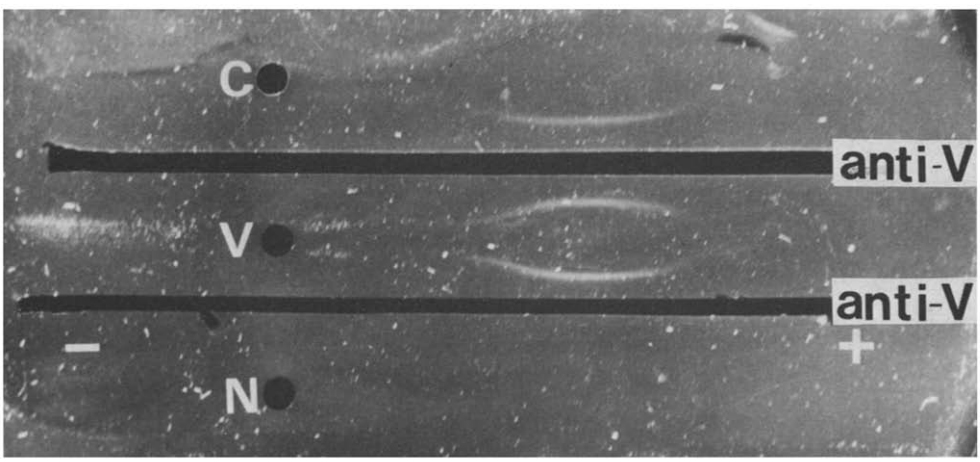

Fig. 7. Immunoelectrophoresis of arylsulfatase A. Both troughs contain IgG raised against voided urine enzyme (anti-V) and the voided urine, and nephrostomic and cystostomic enzymes were served to immunoelectrophoresis. Each protein concentration was 10.5 mg/ml.

The fact that IgG raised against voided urine enzyme reacted only with the voided urine enzyme and not with the nephrostomic enzyme was established by urine enzymes from three other patients with congenital stenosis of the pelvi-ureteric junction.

Discussion

Arylsulfatase A in voided human urine has been purified and studied in detail by some investigators [9,10]. In this study urine was also collected from another urinary tract and the nature of arylsulfatase A from this different urine origin was compared in the same individuals.

The general enzymatic properties of the voided urine and nephrostomic enzymes differed significantly with respect to the isoelectric point, heat stability and the kinetic parameters, especially the V values. Furthermore, both enzymes differed completely in their immunological reactivity. A second form of arylsulfatase A in human urine has been observed by Stevens et al. [24] although their investigation was performed only with voided urine, two forms of arylsulfatase A_α for the normal or predominant form, and arylsulfatase A_β for the new or second form were observed. The A_α and A_β showed similar activity toward the nitrocatechol sulfate and the same immunological reactivity. In addition, the rate of heat inactivation did not reveal any differences between the A_α and A_β and upon isoelectric focusing, both the α- and β-enzymes exhibited the same pI values near 4.7. These results show that the arylsulfatase A_β, as reported by Stevens et al. [24], is not the same variant form as the nephrostomic enzyme in this communication.

Altered forms of arylsulfatase A have been observed by others [25]. Goldstone et al. reported that arylsulfatase A was converted to electrophoretically different forms by treatment with a bacterial neuraminidase [25]. Since the voided urine and nephrostomic enzymes, like the ox liver enzyme [26], are glycoprotein in nature judging from their affinity for Con A-Sepharose, the enzymatic and immunological differences of both the enzymes might be due to a different carbohydrate chain in the enzymes.

The major percentage of the protein in urine is albumin; the tenacious contamination of urinary arylsulfatase A by albumin has been noted by Stevens et al. [10]. In the present study, an immunochemical evaluation confirmed the separation of the purified arylsulfatase A from albumin because no precipitin line between the IgG antibody and albumin was detected.

Boyland et al. [5], have reported that ureteric urine shows arylsulfatase A activity. Although some of the nitrocatechol sulfate hydrolyzing activity of urine may be derived from the cells of urinary and genital tracts, these factors apparently contribute little to arylsulfatase A activity, since there are few differences between whole and centrifuged urines [5]. Boyland et al. found no correlation between the enzymatic activity and the red and white and epithelial cell counts in normal urine.

The function of the variant form of arylsulfatase A extracted from the urine of the nephrostomic tube, and how the nephrostomic enzyme is changed into the voided urine enzyme during transport in the urinary tract are important questions which merit further investigation.

Acknowledgements

We are indebted to Dr. Shinsei Gasa for his assistance in immunization of the rabbit samples and to Miss Junko Natsuhori for her typing of this manuscript.

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