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Studies on Human Urinary and Renal Esterases That Migrate to the γ -Globulin Region upon Cellulose Acetate Electrophoresis¹⁾

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Large amounts of an esterase that migrated to the γ -globulin region (γ -esterase) upon cellulose acetate electrophoresis were excreted in the urine from patients with renal diseases. The γ -esterase localized in the renal tubules of the kidney in patients with renal tubular diseases was purified and shown to be identical with the urinary esterase that migrated to the γ -globulin region by immunological studies. The esterase has a molecular weight of 66000, an optimum pH of 7.5, and pI values of 7.9 and 8.3.

Keywords— γ -esterases from human urine and kidney; glomerular nephritis; tubular nephritis; substrate specificity; immunoelectrophoresis

Introduction

Esterases found in the sera of animals can be classified into three main types;²⁻⁴⁾ arylesterases which hydrolyze aromatic esters, aliesterases which preferentially hydrolyze aliphatic esters, and cholinesterases which hydrolyze choline esters at higher rates than either aliphatic esters or aromatic esters. Aliesterase capable of hydrolyzing carboxyl esters, thiol esters and aromatic amides is present in most animal tissues.^{5,6)} There are, however, only a few reports on esterases of human kidney and urine.^{7,8)}

It is now well known that various proteins are excreted in the urine of patients with renal diseases, particularly glomerular and tubular nephritis.^{9,10)} Two types of proteins with esterase activity were detected in human urine by starch gel electrophoresis, as described by Terrien *et al.*¹¹⁾ We discovered that an esterase that migrated to the γ -globulin region (γ -esterase) upon cellulose acetate electrophoresis is excreted in the urine of patients with renal diseases. In a preliminary experiment, we found that the esterase in human kidney exhibited the same mobility as this γ -esterase, so it was suggested that the amount of γ -esterase excreted in the urine may reflect the degree of damage of the kidney tissue. The assay of this enzyme in human urine could therefore represent an important clinical test for the diagnosis of renal diseases.

In this work, γ -esterase from human kidney was purified, and its enzymic and immunological properties were investigated.

Materials and Methods

Materials—DEAE-cellulose and CM-cellulose were obtained from Whatman Ltd., Sephadex G-150 and CM-Sephadex C-50 from Pharmacia Fine Chemicals, 4-aminoantipyrine and α -naphthyl acetate from Wako Pure Chemical Industries, Ltd., phenylmethylsulfonyl fluoride from Sigma Chemical Co., and Cellogel from Seikagaku Kogyo Co., Ltd. Other chemicals were of analytical reagent grade.

Preparation of Crude Enzyme from Human Kidney—All steps were carried out at 0 to 4°C. Human kidney (6000 g) was homogenized with 3 volumes of distilled water and 1 volume of *n*-butanol in a Polytronhomogenizer, and the homogenate was centrifuged (10000 $\times g$, 20 min). To the soluble fraction, 1.5 volumes of cold acetone was added. The resulting precipitate was dissolved in 10 mM Tris-HCl buffer (pH 7.5), and this solution was used as the crude enzyme.

Assay of Esterase Activity—The reaction mixture consisted of 2 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM 4-aminoantipyrine, 10 μ l of 10% Triton X-100, and 50 μ l of 53.7 mM α -naphthyl acetate dissolved in ethanol. The reaction mixture was preincubated at 37°C for 5 min, then the enzyme solution was added, and the whole was incubated at 37°C for 10 min. The enzyme reaction was stopped by the addition of 0.1 ml of 20 mM phenylmethylsulfonyl fluoride in ethanol. Color was developed by the addition of 2 ml of 3 mM potassium ferricyanide and 130 mM boric acid dissolved in 50 mM Tris-HCl buffer (pH 7.4). The absorbance at 500 nm was measured. One unit of esterase was defined as the amount which was able to produce 1 μ mole of α -naphthol per min under the above conditions, and specific activity was expressed as units per mg of protein.

Assay of Amidase Activity—Amidase activity was determined by minor modifications of the method of Hiwada *et al.*¹²⁾ using β -naphthylamide derivatives as substrates. A 2 ml aliquot of 0.4 mM substrate in 50 mM Tris-HCl buffer (pH 8.0) was preincubated at 37°C for 5 min. The enzyme reaction was started by the addition of the enzyme, and the mixture was incubated at 37°C for 10 min. The reaction was stopped by the addition of one ml of 4.2 M acetic acid in ethanol, then one ml of the color reagent (11.4 mM *p*-(dimethyl-amino)cinnamaldehyde in ethanol) was added. The reaction mixture was incubated at 37°C for 20 min. The absorbance at 535 nm was measured.

Protein—Protein was determined by the method of Lowry *et al.*¹³⁾ with bovine serum albumin as a standard and by measuring the absorbance at 280 nm.

Disc Electrophoresis—Disc electrophoresis was performed using 7.5% polyacrylamide gel with 50 mM Tris-glycine buffer (pH 9.4) at 3 mA constant current per tube and 4°C for 90 min according to the method of Davis.¹⁴⁾ The gels were stained for protein with Amido Black 10B and for activity with 20 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 ml of α -naphthyl acetate in ethanol (10 mg/ml) and 2 mg of Fast Blue BB salt.

Isoelectrofocusing—Isoelectrofocusing was carried out as described by Vesterberg and Svensson,¹⁵⁾ using 1% carrier ampholyte (pH 3–10) at a constant voltage of 800 V for 48 h.

Electrophoresis on Cellulose Acetate—Electrophoresis on cellulose acetate strips was performed using Cellogel (Seikagaku Kogyo Co., Ltd., 5 \times 6 cm) by the method of Kohn.¹⁶⁾

Immunoelectrophoresis—Immunoelectrophoresis was performed using 1% agar gel (2.5 \times 7.5 cm) by the method of Grabar and Williams.¹⁷⁾

Preparation of Antibody—Female rabbits were immunized with the purified enzyme emulsified in Freund's complete adjuvant and were injected into each foot pad, as well as subcutaneously. The immunization procedure was repeated subcutaneously at intervals of two weeks. Approximately six weeks after the first injection, the animal were bled and the antibody was purified by the method of Fahey.¹⁸⁾

Photomicrography of Human Kidney Specimen—Photomicrography of a specimen from the kidney of a patient with tubular disease was performed by the method of Hintz and Goldberg.¹⁹⁾ The specimen was stained with fluorescein isothiocyanate-labelled anti-human kidney γ -esterase IgG.

Results

Purification of Human Kidney Esterase

The crude enzyme was brought from 40 to 50% ammonium sulfate saturation by the addition of the solid salt, followed by centrifugation. The resulting precipitate was dissolved in a small volume of 10 mM Tris-HCl buffer (pH 7.5). This enzyme solution was dialyzed against the same buffer for 24 h, and then applied to a DEAE-cellulose column (3 \times 25 cm) previously equilibrated with the same buffer. γ -Esterase that passed through this column was dialyzed against 10 mM phosphate buffer (pH 6.5), and absorbed on a CM-cellulose column (3 \times 25 cm) equilibrated with the same buffer. The column was washed with the same buffer, and the absorbed enzyme was eluted with a linear gradient of 0 to 0.3 M sodium chloride as shown in Fig. 1. The fractions showing enzymic activity were collected and concentrated with a membrane filter (PM-10), then the above buffer was exchanged for 10 mM phosphate buffer (pH 7.0) containing 0.1 M sodium chloride. This enzyme solution was applied to a Sephadex G-150 column (4 \times 90 cm). Equilibration of the column and elution of the enzyme were performed with the same buffer. The chromatogram is shown in Fig. 2. The active fractions were pooled and dialyzed against 20 mM phosphate buffer (pH 6.5) overnight. The dialyzed solution was applied to a CM-Sephadex C-50 column (3 \times 20 cm) equilibrated with the same buffer. The column was washed with the same buffer, and the absorbed enzyme was eluted with a linear gradient of 0 to 0.3 M sodium chloride as shown in Fig. 3. The fractions showing enzymic activity were collected and dialyzed against 10 mM Tris-HCl buffer (pH 7.5). The above

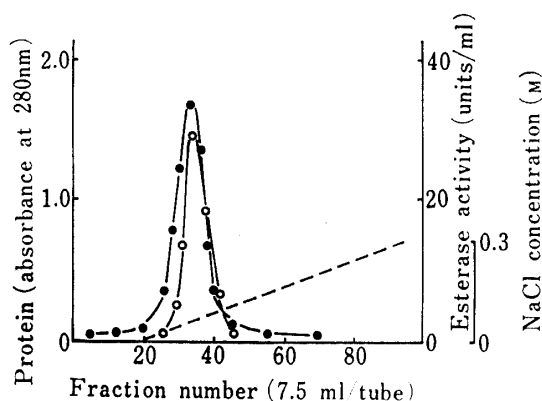


Fig. 1. Column Chromatogram of γ -Esterase from Human Kidney on CM-32 (CM-cellulose)

The DE-52 fractions were applied to a CM-32 column (3×25 cm) equilibrated with 10 mM phosphate buffer (pH 6.5).

The enzyme was eluted with a linear NaCl gradient from 0 to 0.3 M. —●—, protein; —○—, activity; ----, NaCl concentration.

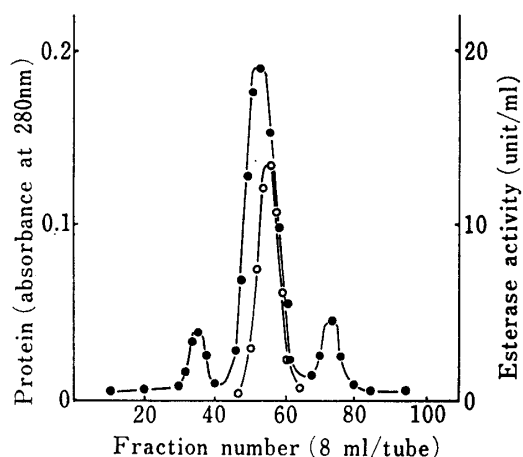


Fig. 2. Column Chromatogram of γ -Esterase from Human Kidney on Sephadex G-150

The CM-32 fractions were concentrated to 10 ml and applied to a Sephadex G-150 column (3.5×90 cm) equilibrated with 10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl.

—●—, protein; —○—, activity.

purification procedures are summarized in Table I. γ -Esterase was purified approximately 430-fold from the crude enzyme with a recovery of 3.94%. As shown in Fig. 4, the peaks of esterase activity coincided with the protein peaks.

Effect of pH on the Activity and Stability

The optimum pH of this enzyme was investigated with Britton-Robinson buffer having various pH values. As shown in Fig. 5, the optimum pH was above 7.5. For the purpose examining pH stability, the enzyme was incubated with buffers having various pH values at 37°C for 30 min and found to be stable over a pH range of 7.0 to 9.0.

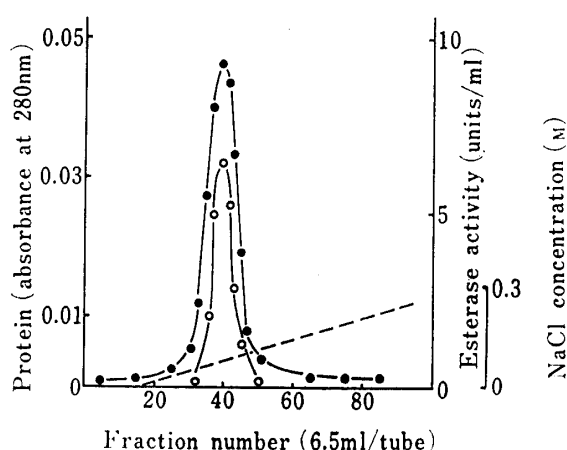


Fig. 3. Column Chromatogram of γ -Esterase from Human Kidney on CM-Sephadex C-50

The Sephadex G-150 fractions were applied to a CM-Sephadex C-50 column (3×20 cm) equilibrated with 20 mM phosphate buffer (pH 6.5). The enzyme was eluted with a linear NaCl gradient from 0 to 0.3 M. —●—, protein; —○—, activity; ----, NaCl concentration.

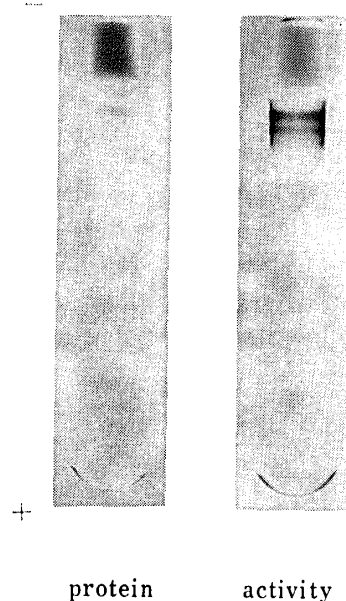


Fig. 4. Disc Electrophoresis of γ -Esterase from Human Kidney
For details, see the text.

TABLE I. Purification of γ -Esterase from Human Kidney

Step of purification	T.P. ^{a)} (mg)	T.A. ^{b)} (units)	S.A. ^{c)} (units/mg)
Crude	93800	13700	0.15
DE-52 (DEAE-cellulose) column chromatography	1810	6490	3.58
CM-32 (CM-cellulose) column chromatography	290	3030	10.5
Sephadex G-150 column chromatography	35.0	1270	36.1
CM-Sephadex C-50 column chromatography	8.60	540	62.9

a) T.P.: Total protein.

b) T.A.: Total activity.

c) S.A.: Specific activity.

Effect of Temperature on the Activity and Stability

For the examination of heat stability, the enzyme was kept at various temperatures and pH 7.5 for 30 min, and the remaining activity was measured in the standard assay system. The purified enzyme retained more than 90% of the maximum activity after treatment at 25°C for 30 min. The optimum temperature for the purified enzyme was 37°C.

Michaelis Constant

The effect of substrate concentration on the purified enzyme was examined. The K_m value was calculated graphically by means of a Lineweaver-Burk plot. As shown in Fig. 6, the apparent K_m value for α -naphthyl acetate was 0.64 mM.

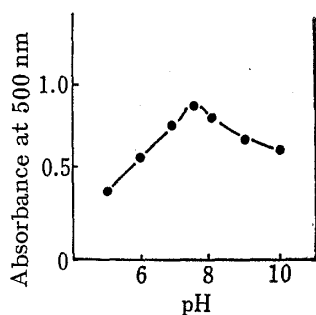


Fig. 5. Optimum pH of γ -Esterase from Human Kidney

The enzyme activity was measured by the standard method using Britton-Robinson buffer (pH 4–10).

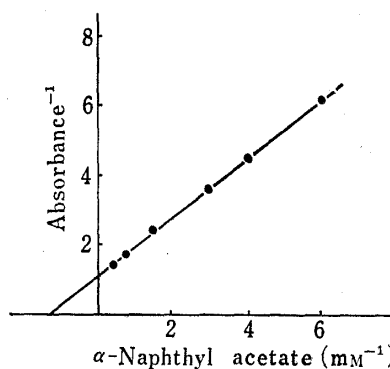


Fig. 6. Lineweaver-Burk Plot of γ -Esterase from Human Kidney

γ -Esterase activity assayed by the standard method using α -naphthyl acetate as a substrate.

Effects of Various Metal Salts and Reagents on the Activity

γ -Esterase activity was measured in the presence of metal salt or reagent. γ -Esterase from human kidney was strongly inhibited by diisopropyl fluorophosphate, but was not inhibited by eserine sulfate, as shown in Table II.

Substrate Specificity

Hydrolysis of a series of esters of α -naphthol, β -naphthol, p -nitrophenol or β -naphthylamide by the purified enzyme was investigated. As can be seen from Table III, α -naphthyl acetate and p -nitrophenyl propionate were hydrolyzed rapidly.

TABLE II. Effects of Various Metal Salts and Reagents on the Activity of γ -Esterase from Human Kidney

Metal salt (1 mM)	Remaining activity (%)	Reagent (1 mM)	Remaining activity (%)
None	100.0	None	100.0
NaCl	93.0	<i>o</i> -Phenanthroline	99.4
CaCl ₂	93.4	EDTA	99.7
MgCl ₂	89.0	Monoiodoacetate	89.7
ZnCl ₂	73.8	Iodine	77.2
CdCl ₂	95.6	N-Bromosuccinimide (NBS)	55.4
CuCl ₂	92.9	Cysteine	103.9
NiCl ₂	90.6	Dithiothreitol	102.9
MnCl ₂	101.6	2-Mercaptoethanol	100.4
CoCl ₂	93.4	DFP ^{a)} (0.3 mM)	16.1
FeCl ₂	83.0	Eserine sulfate	100.0
HgCl ₂	81.3	PCMB ^{b)} (1.3 mM)	55.5
		KCN (1.3 mM)	95.6

a) Diisopropyl fluorophosphate.

b) *p*-Chloromercuribenzoate

The enzyme was incubated with a metal salt or reagent in 10 mM Tris-HCl buffer (pH 7.5) at 37°C for 30 min. The incubation mixture was diluted 10-fold with 10 mM Tris-HCl buffer (pH 7.5), and the remaining activity was assayed by the standard method.

TABLE III. Substrate Specificity of γ -Esterase from Human Kidney

Substrate	Relative activity (%)	Substrate	Relative activity (%)
α -Naphthyl acetate	100.0	<i>p</i> -Nitrophenyl acetate	7.2
α -Naphthyl propionate	8.7	<i>p</i> -Nitrophenyl propionate	309.4
α -Naphthyl butyrate	0.6	L-Alanyl- β -naphthylamide	0
α -Naphthyl valerate	0	L-Valine- β -naphthylamide	0
α -Naphthyl caproate	0	L-Leucine- β -naphthylamide	0
β -Naphthyl acetate	52.3	L-Seryl- β -naphthylamide	0
β -Naphthyl propionate	8.8	L-Cysteine- β -naphthylamide	0
β -Naphthyl butyrate	2.0	L-Prolyl- β -naphthylamide	0
β -Naphthyl valerate	0		
β -Naphthyl caproate	0		
β -Naphthyl laurate	0		
β -Naphthyl oleate	0		

The substrate specificity of γ -esterase from human kidney was assayed by the standard method described in the text. The values were calculated as percentages of α -naphthyl acetate hydrolysis.

Isoelectric Point

The isoelectric points of this enzyme were pH 7.9 and 8.3.

Determination of the Molecular Weight of the Purified Enzyme

The molecular weight of the purified enzyme was determined by gel filtration on Sephadex G-150 according to the method of Andrews,²⁰⁾ and was found to be 66000 as shown in Fig. 7.

Electrophoretic Patterns on Cellogel of Human Serum, Liver, Urine and Kidney Esterases

Human liver was homogenized with distilled water. The results (Fig. 8) showed that γ -esterase was not detectable in human serum or liver. γ -Esterases in human kidney and urine from a patient with renal disease exhibited the same mobility toward the cathodal region on Cellogel. Therefore, human urinary γ -esterase may originate from the kidney.

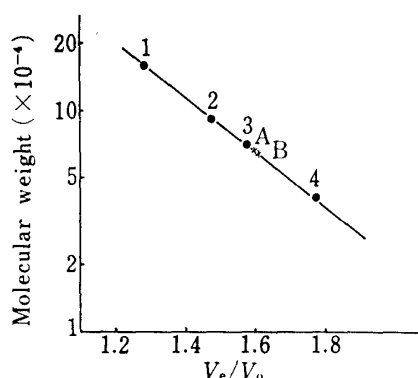


Fig. 7. Molecular Weight of γ -Esterase from Human Urine and Kidney as determined by Sephadex G-150 Gel Filtration

The samples were applied to a Sephadex G-150 column (2×65 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl. A, γ -esterase from urine of a patient with renal disease; B, γ -esterase from human kidney; 1, γ -globulin (M.W. 16.0×10^4); 2, phosphorylase a (M.W. 9.4×10^4); 3, bovine serum albumin (M.W. 6.7×10^4); 4, ovalbumin (M.W. 4.3×10^4)

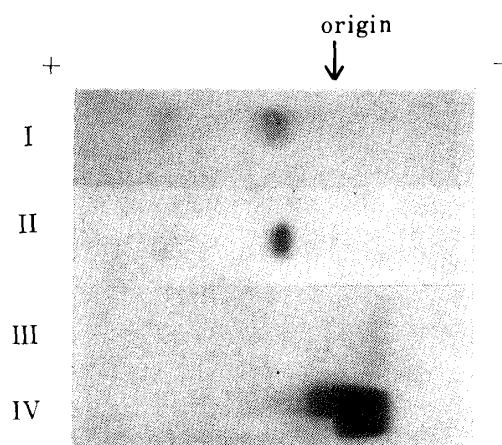


Fig. 8. Electrophoretic Patterns of Non-specific Esterases and γ -Esterase

The electrophoresis was run at 0.8 mA/cm for 50 min. Esterase activity was stained with α -naphthyl acetate and Fast Blue BB salt. I, human serum; II, human liver; III, urine from a patient with renal disease; IV, human kidney.

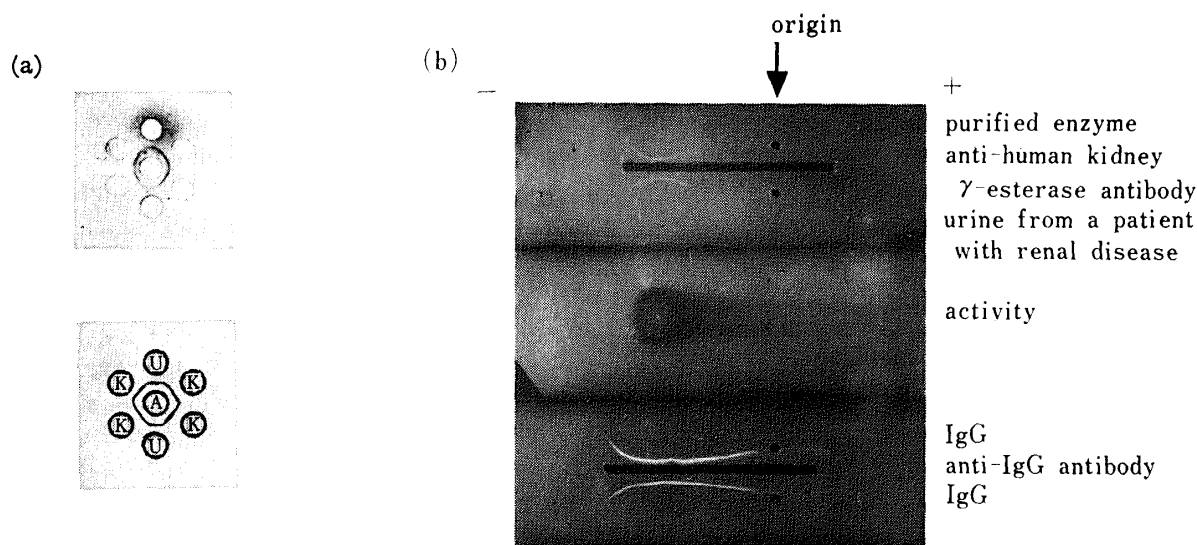


Fig. 9. Immunodiffusion (a) and Immunoelectrophoresis (b) of γ -Esterase against Anti-human kidney γ -esterase antibody

(a) A, anti-human kidney γ -esterase antibody;
K, γ -esterase from human kidney;
U, urine from a patient with renal disease.

Immunological Studies of Human Urinary and Kidney γ -Esterases

Ouchterlony immunodiffusion analyses with anti-human kidney γ -esterase antibody are shown in Fig. 9(a). The results indicated complete immunological identity between human kidney and urinary γ -esterases on Ouchterlony immunodiffusion analyses with a specific anti-human kidney γ -esterase antibody. The precipitate lines of human kidney and urinary γ -esterases fused completely, and upon treatment with anti-human kidney γ -esterase antibody, the activity of urinary γ -esterase on polyacrylamide gel completely disappeared. Therefore, γ -esterases from human urine and kidney are immunologically identical. However, a similar experiment showed that human liver esterase was not identical with our purified enzyme.

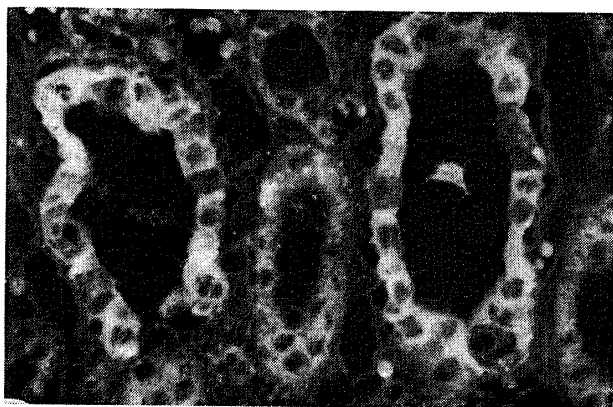


Fig. 10. Photomicrograph of a Biopsy Specimen from the Kidney of a Patient with Pyelonephrosis

The specimen was stained with fluorescein isothiocyanate-labelled anti-human kidney γ -esterase IgG (rabbit).

In immunoelectrophoretic experiments, γ -esterase from the urine of a patient with renal disease had the same mobility as the purified enzyme. Fig. 9(b) shows that γ -esterases from the urine of a patient with renal disease and from human kidney both migrated to the γ -globulin region.

Photomicrography of a Biopsy Specimen from the Kidney of a Patient with Pyelonephrosis

A specimen from the kidney of a patient with pyelonephrosis was investigated photomicrographically using fluorescein isothiocyanate-labelled anti-human kidney γ -esterase IgG (rabbit). As shown in Fig. 10, the results showed that γ -esterase was

localized in the renal tubules of human kidney. In the case of normal subjects, this enzyme was not detected at that site.

Discussion

γ -Esterase purified from human kidney was partially inhibited by oxidizing reagents, iodine and NBS, and strongly inhibited by DFP. The purified enzyme showed the highest activity toward α -naphthyl acetate and p-nitrophenyl propionate, and only slightly hydrolyzed α - and β -naphthyl butyrate. Furthermore, substrates with a chain length of more than four carbons were not hydrolyzed by this enzyme. γ -Esterase activity was not detected with acetylcholine ester as a substrate. Therefore, the γ -esterase from human kidney is an aliesterase (EC 3.1.1.1) as judged from the substrate specificity and inhibition by DFP and eserine sulfate. This enzyme was very unstable to heat. The urinary and kidney γ -esterases were identical as judged by immunoelectrophoresis. The isoelectric points of this enzyme were pI 7.9 and 8.3. In immunodiffusion analysis, the purified enzyme and the enzyme in urine from patients with renal disease showed only a single precipitation line against anti-human γ -esterase antibody. In addition, in immunoelectrophoresis, the enzyme and the urine from patients with renal disease showed again one single precipitation line with the same mobility against anti-human kidney γ -esterase antibody. These observations suggest that this enzyme has microheterogeneous structure, as reported for urinary kalikrein by Matsuda²¹⁾ and Ikekita.²²⁾ The source of the urinary enzyme is presumably the kidney. Since the enzyme was not detected in renal tubules of normal subjects by immunocytochemical analysis, it may be possible to use this enzyme as an effective marker for renal disease in clinical tests. Further studies are in progress on the properties of the urinary enzyme and on the relation between this enzyme and renal disease.

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