THE HISTOCHEMISTRY OF INDOXYLESTERASE OF RAT KIDNEY WITH SPECIAL REFERENCE TO ITS CATHEPSIN-LIKE ACTIVITY

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CHANGES affecting the hydrolytic enzymes of the nephron in various metabolic disorders have been studied histochemically by a number of authors (Wachstein, 1955; Wachstein and Meisel, 1957; Macpherson and Pearse, 1957; Pearse and Macpherson, 1958). The characteristics of the renal carboxylic acid esterases in particular, however, have not been properly established. Using various α-naphthyl substrates and various inhibitors, Gomori (1955) obtained evidence of an eserine-resistant carboxylic acid esterase in human kidney. This enzyme was partially inhibited by organophosphorus compounds and showed some of the features of a lipase. From the work of Aldridge (1954) it became evident that the use of a variety of substrates was less successful in differentiating esterase activities than the use of various activators and inhibitors. Although a number of naphthyl and indoxyl substrates are available for histochemical use, distinction between the different types of esterase is probably better made by the use of a single substrate. Advantage can be taken, therefore, of the precise intracellular localization offered by the halogen-substituted indoxyls (Holt, 1956).

Aldridge (1954) separated the carboxylic acid esterases into two types (A and B) by their sensitivity towards organophosphorus inhibitors. In the present study, advantage was taken of the fact that E600 (diethyl-*p*-nitrophenylphosphate) inhibits irreversibly the B-type esterases only, allowing the properties of the A-type to be investigated. Histochemical studies (Pearse, 1956; Pepler and Pearse, 1957*a* and 1957*b*; Pearse and Macpherson, 1958) of the indoxyl esterases of hypophysis, brain, thyroid and kidney suggested that part of the observed esterolytic activity was due to a proteolytic enzyme, since several of these enzymes are known (cf. Neurath and Schwert, 1950) to be capable of splitting esters as well as peptides. In the present paper it will be shown that in fact a cathepsin-like enzyme is partially responsible for the histochemical demonstration of A-type esterase by the indoxyl method.

MATERIALS AND METHODS

Stock hooded male rats, weighing 200–350 g. were used throughout. The animals were killed by decapitation and the kidneys, part of the liver, and the pancreas were removed and fixed for approximately 24 hr. in cold (4°) formalin-calcium. Sections were cut on a freezing microtome at 7·5 μ, and after brief washing in distilled water were used for esterase studies. For the demonstration of leucine aminopeptidase, fresh frozen sections, cut at 8–15 μ on a remotely-controlled cold microtome (cryostat), were used.

The esterase method was performed according to Holt and Withers (1952) using *o*-acetyl-5-bromoindoxyl as a substrate. Free-floating sections were incubated for 2 hr. at 37°. In the pH studies *o*-butyryl indoxyl was tested in addition to the acetate.

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In order to compare the esterase activity with that shown by a substrate specific intracellular proteolytic enzyme, we used the method of Nachlas, Crawford and Seligman (1957) for the demonstration of leucine aminopeptidase (pH 6·5) with L-leucyl-β-naphthylamide hydrochloride as a substrate.

The B-type esterases were eliminated by incubation of the sections in 10^{-5} M E600 (diethyl-*p*-nitrophenylphosphate) in buffer for 1 hr. at 37°. This treatment resulted in an approximately two-thirds loss of esterase activity, except in certain sites referred to below. In control studies, 10^{-3} M Mipafox (NN′-di-*iso*-propylphosphorodiamidic fluoride) was shown to have the same effect as 10^{-5} M E600. After this treatment the sections, containing only E600-resistant enzyme, were transferred to solutions of various activators and inhibitors (see Table) in buffer pH 5·3 and incubated for 1 hr. Thereafter the tissues were immersed in the substrate mixture at pH 5·3, containing the appropriate activator or inhibitor.

TABLE.—*Inhibition and Activation of E600-resistant Rat Kidney Esterase*

Substance	Concentration	Proximal tubules	Distal tubules*	Epithelium of the papilla
Ascorbic acid	2×10^{-3} M	+1	+1	+1
Cysteine	1×10^{-3} M	+1	+1	0
Glutathione	1×10^{-3} M	+1	+1	0
β-phenylpropionic acid	1×10^{-2} M	−2	−2	−2
D-L phenylalanine	1×10^{-3} M	−1	−1	−1
Sodium citrate	1×10^{-2} M	−1	−1	−1
Sodium fluoride	1×10^{-3} M	−1	−1	0
Iodoacetic acid	5×10^{-3} M	−1	−	−2
Potassium cyanide	2×10^{-3} M	−1	−1	−1
Magnesium chloride	5×10^{-2} M	0	0	0
Cupric sulphate†	1×10^{-3} M	−1	−1	−1
Lead nitrate†	1×10^{-3} M	−2	−2	−2
2 : 4 dinitro-1-fluorobenzene	1×10^{-3} M	0	0	0
Tween 60	10^{-3} g./100 ml.	+2	+2	+1
Tween 80	10^{-3} g./100 ml.	+2	+2	+1
Trypsin cryst.‡	1/2 sat. soln.	+2	+2	+2
Phenylmercuric chloride	1×10^{-4} M	+1	+1	0

* Strongly esterase positive intercalated cells.

† Only pre-incubation used, substance not added to substrate.

‡ Pre-incubation in trypsin pH 7·8 at 25° for ½ hr.

Arbitrary grading of enzyme activity—estimated visually, using the following figures: −, inhibition; +, activation; 1 = 25 per cent; 2 = 50 per cent—compared with control sections showing zero activity.

The inhibitor and activator studies were also carried out with control sections.

To ensure the absence of bacteria or fungi, which possess enzymes capable of hydrolyzing indoxyl esters, heated or freshly prepared Veronal-HCl buffer was used throughout.

In vitro studies were performed with crystalline pancreatic chymotrypsin (Bios Laboratories Inc.), trypsin and pepsin (Nutritional Biochemicals Corporation), and carboxypeptidase (L. Light & Co.), obtained commercially and used without further purification. In addition, a crude preparation of papain was tested. Different enzyme concentrations were prepared in ice-cold veronal-HCl buffer (5×10^{-2} M) at the optimal pH of the enzyme under investigation. In the case of carboxypeptidase, the ionic strength of the solutions was maintained with NaCl at 0·2. The concentration of the substrate (*o*-acetyl indoxyl) was restricted by the low solubility of this compound in water to 6×10^{-3} (*cf.* Underhay, 1957). Incubation was carried out at 25° or at 37°. One ml. of the reacting mixture was withdrawn every 15 min. and was vigorously shaken with chloroform for 10 min. The indigotin was extracted after centrifugation. The chloroform was allowed to evaporate in the dark, the dye was redissolved in NN′-dimethylformamide and measured immediately in a spectrophotometer. The enzyme concentration was determined in terms of nitrogen per ml. by the micro-Kjeldahl method.

RESULTS

Histochemical Methods

pH-series

It was found that in rat kidney, esterase activity exhibits two pH-optima, one from pH 4·6 to 6·0 and a second from pH 7·0 to 7·5. This finding suggests the existence of at least two enzymes capable of splitting *o*-acetyl-5-bromoindoxyl. Using *o*-butyryl indoxyl, only one pH-optimum (7·4 to 7·7) was detected. The cleavage of *o*-acetyl-5-bromoindoxyl occurred, in a moderate degree, at both pH 2·6 and pH 9·6.

Distribution of the organophosphorus-resistant esterase

Treatment with E600 caused approximately a two-thirds loss of the total indoxyl esterase activity. This effect was easily reproducible and irreversible. The inhibition, however, did not affect all the tissue elements to the same degree. The proximal tubules, as a whole, suffered a loss of esterase activity. The large intracytoplasmatic droplets, which are normally found in older male rats, showed a strongly resistant esterase (Fig. 3). The descending and ascending limbs were almost devoid of activity. No enzyme could be demonstrated in the thin segment and in the collecting ducts of the outer medulla. Interstitial macrophages and certain cells of the distal tubules and collecting ducts of the cortex contain an esterase which shows a striking resistance to E600 (Fig. 1). These tubular cells are intercalated in the rather flat epithelium of the corresponding ducts. They are usually larger than the esterase-free neighbouring cells and protrude into the lumen of the tubule. The esterase is concentrated in the perinuclear rim and the horns of the crescent so formed point away from the lumen. Occasionally, especially in older animals, these cells are desquamated into the lumen. At present the function of these cells of the distal duct is not clear, but it is suggested that they may be of significance in delayed, distal protein resorption (Mayersbach and Pearse, 1956). They are not identical with intercalated cells of the medullary ducts referred to by Oliver *et al.* (1957).

An organophosphorus-resistant esterase was also present in the pelvic epithelium and in cells of the collecting ducts near the tip of the papilla.

The same resistant enzyme activity could be demonstrated in the Kupffer cells of the liver and to a lesser extent in the liver epithelia surrounding the bile canaliculi. In contrast to the islets of Langerhans and the pancreatic duct epithelium, the zymogen granules of the pancreatic acini exhibited only a faint E600-resistant esterase. This is in perfect agreement with the well known inhibition of most of the active pancreatic enzymes by organophosphorus compounds (Jansen, Nutting, Jang and Balls, 1949).

Properties of the organophosphorus-resistant esterase

The pH-optimum of the E600-resistant esterase is pH 5·0 to pH 5·5, at pH 7·0 activity is markedly decreased. The table summarizes the effect of various activators and inhibitors on the E600-resistant esterase activity of rat kidney at

pH 5·3. The esterase of the Kupffer cells, and of the macrophages of the omentum, behaves in a similar manner.

The properties of the organophosphorus-resistant esterase closely resemble those of an intracellular proteolytic enzyme, *i.e.*, a cathepsin. The activation by trypsin (see Fig. 2 and 4) is a common feature of most of the pancreatic proteases (Neurath and Schwert, 1950). This process is presumed to involve the opening of single peptide bonds in the inactive precursor (Neurath and Dixon, 1957). Trypsin was observed to activate an E600-resistant indoxyl esterase in sections of rat pancreas. The precursors of the pancreatic proteolytic enzymes are not sensitive towards organophosphorus inhibitors. The esterase activity of the proteolytic enzymes is referred to below.

The activation of pancreatic carboxypeptidase by detergents has been described by Gorini and Labouesse-Mercouroff (1954). Histochemically, this effect was most marked, but we failed to increase esterase activity by raising the ionic strength of the incubating mixture to 0.62, a procedure referred to by the same authors.

Cysteine, glutathione, and in certain cases ascorbic acid, activate various cathepsins (Smith, 1951). This action of reducing agents is presumably due to opening of the S–S bonds of the enzyme molecule. The participation of SH-groups in the enzymatic process is suggested also by the inhibitory effect of iodoacetic acid and heavy metal ions.

Inhibition by iodoacetate, D-amino-acids and cyanide, is a property of the carboxypeptidases (Elkins-Kaufmann and Neurath, 1948) and these, as metalloenzymes, are also inhibited by citrate (Vallee, 1955). Histochemical attempts to produce inhibition by chelating agents have been unsuccessful, because of interference with the oxidizing catalyst (0·2 M ferricyanide and 0·2 M ferrocyanide) in the substrate mixture. Mg-ions in high concentration were also without effect. Another metalloenzyme, leucine aminopeptidase, showed an entirely different pattern in the rat kidney when compared with the E600-resistant esterase. As pointed out by Nachlas, Crawford and Seligman, only the tubules of the inner cortex are strongly stained. Leucine aminopeptidase survived only a short formalin fixation, but was resistant to organophosphorus inhibitors. Highly purified leucine aminopeptidase from swine kidney shows very little esterase activity (Smith and Spackman, 1955). The ester substrates of carboxypeptidase do not require a hydrogen atom or a peptide nitrogen at the sensitive bond (Snoke, Schwert and Neurath, 1948), but the presence of a free carboxyl group seems to be essential for the action of this enzyme. Therefore, *o*-acetylindoxyl and *o*-acetyl-5-bromoindoxyl are not attacked by pancreatic carboxypeptidase.

The effect of phenylmercuric ions in low concentration suggests the presence of a " C-esterase ", recently described in hog kidney extract by Bergmann, Segal and Rimon (1957).

Inhibition by β-phenylpropionic acid suggests a chymotrypsin-like activity. This inhibition is competitive in nature and it could be demonstrated in the tissues only in relatively high concentrations. Chymotrypsin is stable to the action of formaldehyde and apparently requires no amino groups for its enzymatic activity (Neurath and Schwert, 1950).

The insensitivity of the E600-resistant enzyme activity to heat (60° for 30 min.) parallels that of a salicyl butyrate-splitting " Esterase II " of rat pancreas, described by Myers, Schotte, Boer and Borsje-Bakker (1955).

In vitro *Experiments*

Of the crystalline proteases used, only chymotrypsin was found to hydrolyze *o*-acetylindoxyl at a measureable rate. The results obtained with two different concentrations of chymotrypsin are presented in Fig. 5. The liberated indoxyl, measured as indigotin, its oxidation product, is plotted against the time of the

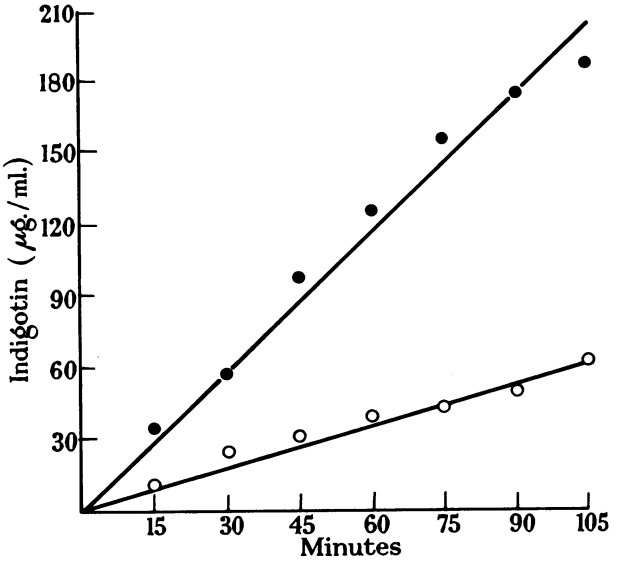


FIG. 5.—Indigotin in micrograms produced by the hydrolysis of *o*-acetyl indoxyl by chymotrypsin at two different concentrations in 5 × 10^{-2} M veronal-HCl buffer at pH 7·8 and 25°. The amount of chymotrypsin nitrogen in these systems is shown by ○ = 0·25 mg. N/ml., ● = 0·44 mg. N/ml. Indigotin measured spectrophotometrically at 590 mμ in N,N′-dimethylformamide.

reaction. The relationship of dye produced to enzyme concentration is a linear one and the reaction is probably of zero order. Our experimental results are not sufficiently accurate to determine the kinetics, however, on account of the low solubility of the substrate in water.

A feeble reaction was obtained with Mg^{++}-activated crystalline trypsin at a comparatively high enzyme concentration. These results were not reliable, however, because bacterial contamination of the reacting medium could not be excluded. A rapid hydrolysis of *o*-acetylindoxyl was achieved with crude papain.

EXPLANATION OF PLATE:

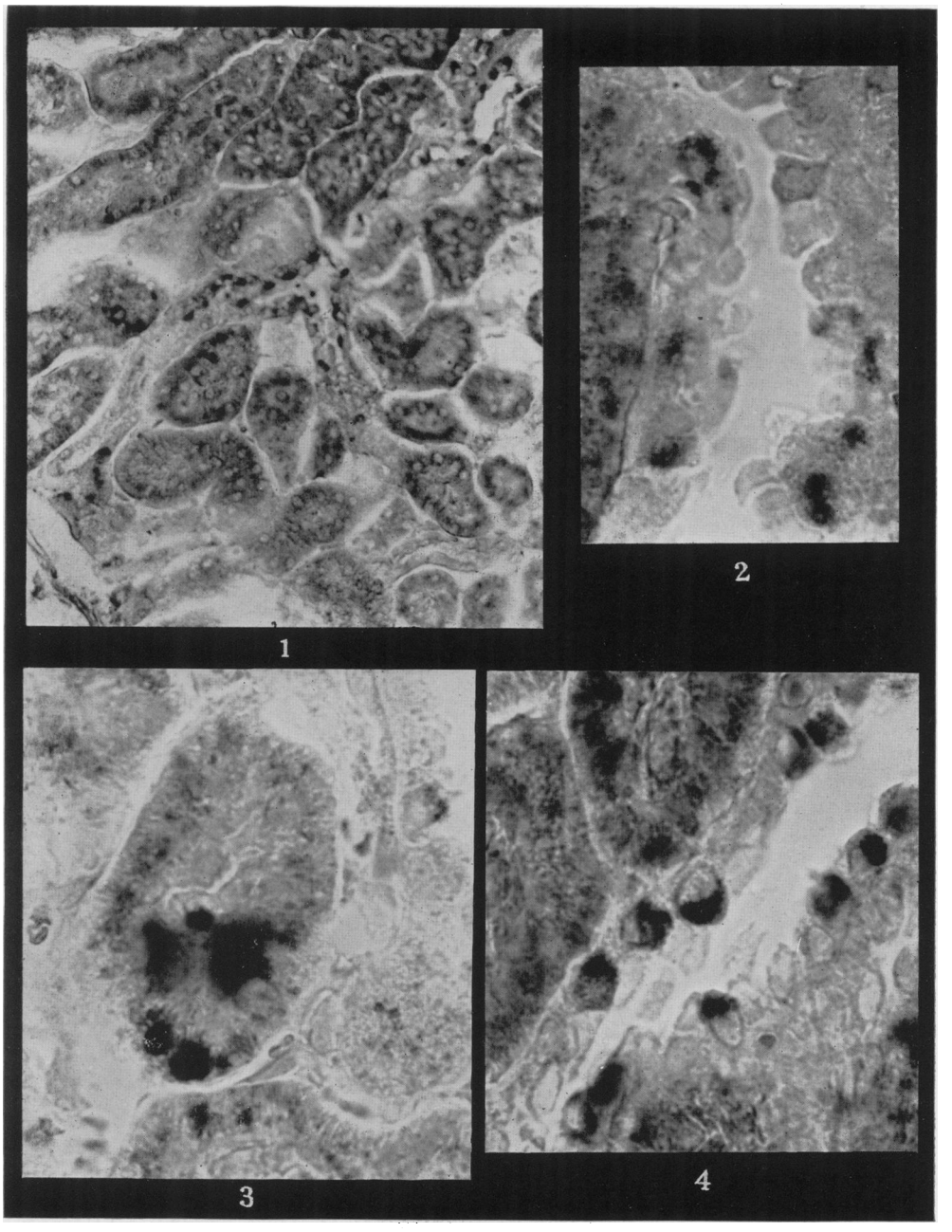
Preparations in Fig. 1–4 are of 7·5 μ cold formol-calcium fixed frozen sections of rat kidney.

FIG. 1.—Part of the outer cortex. The distal tubules and the first part of the collecting tubules show indoxyl esterase activity restricted to intercalated cells (no inhibitor used). × 215.

FIG. 2.—Distal tubule shows E600-resistant indoxyl esterase in intercalated cells (control). × 850.

FIG. 3.—Kidney of old male rat shows E600-resistant indoxyl esterase concentrated in large droplets in the cells of a proximal tubule. × 850.

FIG. 4.—Same kidney as Fig. 3. Activation of E600-resistant indoxyl esterase by pre-incubation with trypsin (½ hour, 25°, pH 7·8). × 850.



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DISCUSSION

In the present paper we describe an organophosphorus-resistant indoxylesterase in the proximal tubules, in certain intercalated cells of the distal tubules and in the pelvic epithelium of rat kidney, which has the characteristics of a cathepsin. A similar enzyme was found in macrophages, in Kupffer cells of the liver, in the islets of Langerhans and in pancreatic duct epithelium. It is probably identical with the non-specific esterase described in pituitary mucoid cells by Pearse (1956), in rat thyroid, rat brain and human argentaffin cell tumours by Pepler and Pearse (1957*b* and 1957*a*) and Pearse and Pepler (1957).

This enzyme system belongs to the A-type esterases (" Aromesterases ") investigated by Aldridge, or to the " Organophosphate-resistant Esterase " of Hobbiger (1957). The differentiation of the esterases is possible only by using specific inhibitors, because the overlap in substrate specificity of these enzymes is considerable (Aldridge). Myers *et al.* (1955) suggested that E600-resistant and heat-resistant esterase of rat pancreas might be related to a proteolytic enzyme, differing from chymotrypsin or trypsin. Histochemically, indoxyl esterase has been shown to have similar properties.

The biochemical evidence for the esterase activity of pancreatic proteolytic enzymes has been reviewed by Neurath and Schwert (1950). This activity, investigated mostly with synthetic amino-acid ester substrates, is exhibited also by cathepsins. These intracellular proteolytic enzymes act on proteins or on synthetic substrates for well characterized proteases and peptidases, but are optimally active at an acid pH. A new classification of the animal cathepsins, based on substrate specificities, was proposed by Tallan, Jones and Fruton (1952). Their " Cathepsin A " is comparable to pancreatic pepsin, " Cathepsin B " to trypsin and " Cathepsin C " to chymotrypsin. In addition, leucine aminopeptidase (and other aminopeptidases), a carboxypeptidase and a tripeptidase were distinguished. The histochemical properties of the E600-resistant esterase investigated are characteristic of cathepsin C. This assumption is strongly supported by the ability of chymotrypsin to hydrolyze *o*-acetylindoxyl. Concerning this substrate, the properties of purified beef spleen cathepsin C are of special interest. This enzyme requires in its substrate a free terminal α-amino or α-imino group. The interaction of cathepsin C with a substrate involves such a group as well as the carbonyl group participating in the sensitive linkage. A primary α-amino group is not essential. Near pH 5 this enzyme shows predominantly hydrolysis, near pH 7·5 predominantly transamidation (Izumia and Fruton, 1956).

With the exception of leucine aminopeptidase, the action of other cathepsins on *o*-acetylindoxyl cannot be wholly excluded at present. Cathepsin B (Greenbaum and Fruton, 1957) must be considered on account of the weak hydrolytic activity of trypsin on *o*-acetylindoxyl and of the activating effect shown by reducing agents. A carboxypeptidase-like action may be deduced from the strong activation by detergents. Organophosphorus compounds do not inhibit purified intracellular proteolytic enzymes. They also fail to influence the precursors of pancreatic proteases in contrast to their strong inhibition of the active enzymes (Jansen *et al.*, 1949). The trypsin-effect on E600-resistant indoxyl esterase suggests that part of this enzyme is present in the cell as a precursor.

A dialkylfluorophosphatase (DFP-ase) (Mounter, Alexander, Tuck and Dien, 1957) seems not to be involved in the enzyme activity described. Hog kidney

DFP-ase acts optimally at pH 8 and is inhibited by dinitrofluorobenzene. On the other hand, the participation of a " C-esterase ", described in hog kidney extract by Bergmann, Segal and Rimon (1957), is most likely. This enzyme differs from the A-esterase of Aldridge and from the DFP-ase of Mounter. It acts optimally at pH 7·4 and is not inhibited by organophosphorus compounds. It is activated by monovalent organic mercurials, an effect which is easily produced with E600-resistant indoxyl esterase. The histochemical evidence, therefore, would suggest a close relationship of C-esterase and a cathepsin, considering the wide pH-range of the latter.

Erythrocytes, plasma and liver esterase hydrolyze *o*-acetylindoxyl at a much higher rate than crystalline chymotrypsin (*cf.* manometric determinations by Underhay, 1957 and Hobbiger, 1957). Quantitative results obtained by purified enzymes or tissue homogenates cannot be related directly to histochemical reactions, since it is not possible to demonstrate a marked difference in enzyme content between various cell types by ordinary biochemical methods.

SUMMARY

The histochemical properties of an organophosphorus-resistant esterase of rat kidney have been studied with *o*-acetyl-5-bromoindoxyl as substrate. The effects of inhibitors and activators, and the determination of the optimal pH, suggest that at least part of this enzymatic action may be due to a cathepsin.

In vitro hydrolysis of *o*-acetylindoxyl by crystalline pancreatic chymotrypsin further suggests that the intracellular proteolytic enzyme involved is probably cathepsin C.

Apart from the kidney, this cathepsin-like activity has been shown in macrophages, Kupffer cells, in various epithelial cells and in the pancreatic islets.

The biochemistry of kidney esterases is discussed in relation to the indoxyl esterase investigated.

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