AMINOPEPTIDASES OF RAT TESTIS

I. FRACTIONATION AND CHARACTERIZATION

TAPANI VANHA-PERTTULA

*Department of Anatomy, University of Turku,
20520 Turku 52, Finland*

(Received 13th December 1971)

Summary. Aminoacyl naphthylamidases or aminopeptidases of the rat testicular tissue were fractionated by DEAE-cellulose chromatography and their subcellular site was evaluated by continuous sucrose gradient centrifugation. Four different enzymes were separated with distinct enzymatic properties.

Enzyme I preferentially hydrolysed methionyl- β -NA followed by valyl-, isoleucyl-, leucyl-, phenylalanyl- and alanyl- β -NA at pH 7.0. A slight activation with cysteine and EDTA and a marked inhibition with heavy metal ions was observed. This enzyme was connected to the particles of the mitochondrial-lysosomal fraction. Its main site of function is presumably within the testicular interstitial tissue.

Enzyme II readily hydrolysed a wide variety of substrates with preference for lysyl- and arginyl- β -NA at pH 6.8. It was markedly activated by chelating agents and sensitive to heavy metal ions as well as to some divalent metal ions. This enzyme was tentatively localized in membranous structures and exclusively in the testicular interstitial tissue.

Enzyme III showed the typical characteristics of aminopeptidase B with a marked activation by halide ions. It hydrolysed only arginyl- and lysyl- β -NA optimally at pH 6.5. It is a soluble enzyme and mainly located within the seminiferous tubules.

Enzyme IV showed a large substrate spectrum with optimum at pH 8.0. It was an SH-dependent soluble enzyme. Its main site was within the seminiferous tubules.

None of the four enzymes appear specific for testicular tissue since similar enzymes have been reported in other tissues. Their physiological significance can be evaluated with differential quantification during experimental conditions.

INTRODUCTION

The plurality of enzymes hydrolysing different aminoacyl naphthylamides in various mammalian tissues has been established (Vanha-Perttula & Hopsu, 1965, 1966; Vanha-Perttula, Hopsu & Glenner, 1966; Ellis & Perry, 1966; Mäkinen & Raekallio, 1969). Most of them have a large substrate spectrum, while a few have a more limited substrate specificity (Glenner, McMillan &

Folk, 1962; Hopsu, Mäkinen & Glenner, 1966a, b). At the present time, hardly anything is known of their physiological function although changes have been observed in their activity with varying functional conditions of tissues (Vanha-Perttula, 1969).

Aminopeptidases with limited substrate specificity have been implicated as specific enzymes for angiotensin hydrolysis (aminopeptidase A; Nagatsu, Nagatsu, Yamamoto, Glenner & Mehl, 1970) and for the formation of bradykinin during the inflammatory reaction (aminopeptidase B; Hopsu-Havu, Mäkinen & Glenner, 1966).

Due to overlapping substrate specificity, the conventional histochemical techniques do not offer a solution for the cellular localization of most of these enzymes. Another reason for the unsuccessful histochemical localization of some of these enzymes is their solubility, e.g. in the ovary (Vanha-Perttula & Hopsu, 1966) and other endocrine tissues (Vanha-Perttula & Hopsu, 1965; Ellis & Perry, 1966). The histochemical reaction in the rat testicular tissue also gives poor results. The enzymatic reaction is diffuse suggesting the presence of soluble enzymes. A slightly stronger reaction is present in the Leydig cells as compared to the seminiferous tubules when different aminoacyl naphthylamides are used as substrate (T. Vanha-Perttula, unpublished observations).

To obtain information on the aminopeptidase content and composition of the rat testis, biochemical fractionation studies were carried out. To discover the possible localization of the enzymes in the testis, the seminiferous tubules were isolated and their enzyme composition was separately analysed.

MATERIALS AND METHODS

Tissue preparation

Testicular tissue was obtained from adult male albino rats. The testicular capsule and visible blood vessels were removed. A total tissue homogenate was made in 0.02 M-tris-HCl buffer, pH 7.5, using, initially, a glass homogenizer with Teflon pestle. Subsequently, the homogenate was sonicated with an MSE Ultrasonic Disintegrator for 30 sec. This and all subsequent procedures were carried out at 4° C. Testicular tubules were isolated under a dissecting microscope in 0.25 M-sucrose solution buffered to pH 7.5 with 0.02 M-tris-HCl. The tubular tissue was then homogenized in the same buffer and subjected to the same fractionation studies as the total testicular homogenate.

To obtain the crude subcellular fractions, the tissue was homogenized in 0.25 M-sucrose in a glass homogenizer. Twenty strokes were made with the Teflon pestle. The nuclei were separated by centrifugation at 600 *g* for 10 min over a layer of 0.35 M-sucrose. The pellet was recentrifuged after repeated homogenization. This pellet was the 'nuclear fraction'. The combined supernatants were centrifuged at 9000 *g* for 20 min. The resulting pellet was also rehomogenized and recentrifuged and formed the 'mitochondrial-lysosomal fraction'. The combined supernatants were centrifuged at 105,000 *g* for 60 min. This pellet was the 'microsomal fraction'. The final supernatant was called the 'soluble fraction'.

Aliquots of the particle fractions were suspended in 0.35 M-sucrose and

subjected to further separation by density gradient centrifugation. The rest of the particle fractions and the soluble fraction were separately submitted to chromatographic fractionation after preliminary sonication in 0.02 M-tris-HCl buffer, pH 7.5.

Sucrose gradient centrifugation

A continuous sucrose gradient was prepared with an MSE gradient mixer and Zero-Max peristaltic pump (Stålprodukter, Stockholm) utilizing the range of 0.5 to 1.5 M-sucrose. The nuclear, mitochondrial-lysosomal and microsomal fractions were carefully layered over the gradient (vol. 18 ml) in a centrifuge tube. The centrifugation was carried out at 55,000 *g* for 3 hr with a swing-out rotor fitted to the MSE Superspeed 50. After centrifugation, 0.8-ml fractions were collected starting from the bottom of the tube.

Chromatography

A DEAE-cellulose (DE 23, Whatman) column was pretreated with 0.5 M-HCl followed by 0.5 M-NaOH. Before preparing the column, the cellulose was well washed and stabilized with 0.02 M-tris-HCl, pH 7.5. Total testicular homogenate, tubular homogenate and homogenates of the crude subcellular fractions were used as enzyme samples in the fractionation. The sample was layered on to the column (2 × 35 cm) and eluted with a continuous NaCl gradient (0 to 0.35 M) buffered with 0.02 M-tris-HCl, pH 7.5. Fractions of 5 ml were collected.

Measurement of enzymatic activity

The aminoacyl naphthylamidase activity of total homogenate as well as of subcellular, density gradient and chromatographic fractions was determined by the method of Takenaka (1964). The yellow colour was measured at 460 nm with a Beckman DB spectrophotometer. A standard curve with β -naphthylamine HCl was used for calibration. The incubation medium contained 0.5 ml buffer solution (0.1 M-tris-HCl, pH 7.0, unless otherwise indicated), 0.25 ml enzyme solution and 0.25 ml substrate solution (1 mM, unless otherwise indicated). The enzyme activity is given as nmol β -naphthylamine liberated/min/mg protein or ml of enzyme solution. The protein content of the samples was measured utilizing the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard. The protein concentration of the chromatographic fractions was scanned by the absorbance at 280 nm.

Reagents

The β -naphthylamides (β -NA) of various amino acids used in the present study were purchased from Mann Research Laboratories (New York) or from Sigma Chemical Company (St Louis). The metal salts and other reagents used in the modifier studies were of analytical grade and obtained from E. Merck AG (Darmstadt).

Studies on enzyme characteristics

The pH-optima for the different enzymes separated by DEAE-cellulose

chromatography were determined in Britton-Robinson buffer series from pH 5.0 to 9.0 with an appropriate aminoacyl naphthylamide as substrate.

Various modifier substances of analytical grade were dissolved in 0.1 M-tris-HCl buffer, pH 7.0, and the enzyme was incubated with the modifier for 30 min at 37° C before the addition of the substrate.

Pooled samples of different enzymes were also used in the determination of

TEXT-FIG. 1. Elution pattern of aminoacyl aminopeptidases from the DEAE-cellulose chromatography of homogenates of total testis and seminiferous tubules. The column (2 × 30 cm) was eluted with a linear NaCl gradient (0 to 0.35 M). The protein contents of the 5-ml fractions were scanned by the absorbance at 280 nm (---). The hydrolysis rates of different substrates are given in nmol/ml per min as follows: ○, Leu-β-NA; ●, Arg-β-NA; △, Val-β-NA.

the substrate specificity in 0.1 M-tris-HCl buffer, pH 7.0. The final substrate concentration in the incubation medium was 0.25 mM.

Electron microscopic control of sucrose gradient fractions

Samples of sucrose gradient fractions containing hydrolytic activity were sedimented on Millipore filters, fixed in 3% glutaraldehyde and prepared for

electron microscopy. The sections were examined in a Siemens Elmiskop IA electron microscope.

RESULTS

Distribution of aminopeptidases in chromatography of testicular tissue

Four different activities hydrolysing various aminoacyl naphthylamides were separated on DEAE-cellulose chromatography. With Leu- β -NA as substrate, three hydrolytic areas were obtained, namely: Enzyme I in Fractions 21 to 33; Enzyme II in Fractions 35 to 44 and the last enzyme (IV) in Fractions 72 to 90 (Text-fig. 1).

With Arg- β -NA as substrate, three activities were also obtained. The first

TEXT-FIG. 2. Effect of pH on the hydrolytic activity of the testicular aminoacyl aminopeptidases separated by DEAE-cellulose chromatography. Britton-Robinson buffer series was used and the actual pH was measured with a glass electrode before the addition of the aqueous substrate solution. Tracings for the different enzymes are: O, Enzyme I with Leu- β -NA; ●, Enzyme II with Leu- β -NA; △, Enzyme III with Arg- β -NA; □, Enzyme IV with Leu- β -NA.

activity coincided with the area for Enzyme II. It was followed by a hydrolytic activity in Fractions 53 to 68, which was called Enzyme III, and an activity coincident with Enzyme IV activity with Leu- β -NA as substrate.

The DEAE-cellulose chromatography of the isolated seminiferous tubules resulted in separation of three enzymatic activities corresponding to those of Enzymes I, III and IV in elution profile and substrate specificity (Text-fig. 1). Enzymes III and IV were clearly the most prominent in the tubular fractionation. This implies that these enzymes were localized within the seminiferous tubules. Enzyme I could be derived from both tubular and interstitial tissue. Enzyme II clearly appeared to be an interstitial enzyme, since no activity was present in the fractionated tubular tissue.

Chromatographic fractionation of the crude nuclear fraction resulted in separation of low activities for all four enzymes, indicating a possible contamination from other fractions. The main activity obtained with the mitochondrial-lysosomal preparation was Enzyme I. Enzymes II to IV gave much lower activity peaks. The microsomal preparation gave no activity for Enzyme I, but a clear activity peak for Enzyme II. Enzymes III and IV were only present in low activity. The soluble fraction showed almost exclusively Enzymes III and IV.

Optimal pH

The pH optima for the four enzymes separated by DEAE-cellulose chromatography of total testicular tissue were determined with Leu- β -NA as substrate

Table 1. The relative substrate specificity of rat testicular aminoacyl aminopeptidases

Substrate (β -naphthylamide)	Enzyme I	Enzyme II	Enzyme III	Enzyme IV
Arginine	2	74	100	83
Lysine	5	100	70	77
Methionine	100	26	0	81
Alanine	17	42	0	100
Leucine	32	41	0	60
Isoleucine	39	9	0	5
Phenylalanine	24	41	0	80
Valine	41	7	0	8
Tyrosine	1	6	0	22
Glycine	0	15	0	7
Tryptophane	4	16	0	16
Histidine	5	48	0	6
Threonine	3	43	0	10
Proline	3	13	0	19
Hydroxyproline	1	5	0	3
Serine	0	12	0	10

Aliquots of pooled enzyme samples were used to test the hydrolysis rates of various aminoacyl naphthylamide substrates. The final buffer concentration was 0.05 M-tris-HCl, pH 7.0, and the substrate concentration was 0.25 mM. The incubation time was 30 min and 60 min to show the linearity of the enzymatic reaction. The relative hydrolysis rates are given as percentages of the best substrate (100%).

for Enzymes I, II and IV and with Arg- β -NA as substrate for Enzyme III. Text-figure 2 shows the maximal hydrolysis for Enzyme I occurred at pH 7.0, for Enzyme II at pH 6.8, for Enzyme III at pH 6.5 and for Enzyme IV at pH 8.0.

Substrate specificity

Pooled samples for Enzymes I to IV were used to study the substrate specificity with different aminoacyl naphthylamides. Table 1 shows that the preferred substrate for Enzyme I was Met- β -NA followed by Val- β -NA, Ile- β -NA, Leu- β -NA, Phe- β -NA and Ala- β -NA, whereas very low activities were obtained with the other substrates tested.

Enzyme II readily hydrolysed most of the substrates tested. It seemed to prefer the naphthylamides of the basic amino acids, but Leu-, Ala-, Phe-, His- and Thr- β -NA were about equally suitable substrates. Ile-, Val- and Tyr- β -NA showed low hydrolysis rates. Enzyme III showed hydrolytic activity only with Arg- and Lys- β -NA as substrates, which was indicative of similarity to aminopeptidase B in other tissues.

Enzyme IV showed the highest hydrolysis with Ala- β -NA, but the basic (Arg, Lys) aminoacyl naphthylamides as well as Met-, Phe- and Leu- β -NA were readily hydrolysed.

Table 2. Percentage change in the activity of rat testicular aminoacyl aminopeptidases in the presence of different modifiers

Modifier	Enzyme I		Enzyme II		Enzyme III		Enzyme IV	
	A	B	A	B	A	B	A	B
Mg	99	97	54	77	96	108	88	89
Al	68	95	45	67	8	48	30	76
Ca	84	86	53	70	86	96	69	91
Mn ²⁺	74	78	36	66	62	80	76	81
Fe ³⁺	67	85	55	74	15	49	31	87
Fe	79	85	20	62	26	35	22	62
Co	16	41	36	64	62	86	40	70
Ni	10	15	29	64	12	46	36	61
Cu	2	9	8	36	9	10	13	25
Zn	6	19	15	39	17	19	15	28
Ag	5	19	8	15	3	11	21	26
Cd	2	9	2	9	7	10	12	26
Sn	76	85	19	50	85	93	95	94
Sb	65	74	27	59	84	96	49	86
Hg	2	7	6	9	6	7	23	60
Pb	14	19	22	46	13	55	13	49
EDTA	123	124	273	225	81	94	82	96
Cysteine	122	126	104	104	111	92	183	126
p-Chloromercuribenzoate	6	28	6	10	10	20	23	47
α , α' -Dipyridyl	46	52	121	105	74	91	94	96
Ascorbic acid	94	95	110	106	105	98	88	88
1,10-Phenanthroline	112	110	251	213	28	81	25	93

Aliquots of pooled enzyme samples were preincubated with the modifiers at two concentrations for 30 min at 37° C before the addition of the substrates. Val- β -NA was used for Enzyme I, Arg- β -NA for Enzyme II, Arg- β -NA for Enzyme III and Leu- β -NA for Enzyme IV. The final buffer concentration was 0.05 M-tris-HCl, pH 7.0. The change in the hydrolysis is given as a percentage of the controls (100%). The two modifier concentrations were 1 mM (A) and 0.1 mM (B).

Modifier characteristics

Table 2 shows the percentage change of the enzymatic activity in the presence of two concentrations of various modifier substances. To minimize the contamination from the neighbouring enzyme, the substrates were chosen according to their specificities. Enzyme I activity was determined with Val- β -NA as substrate, which is only minimally hydrolysed by Enzyme II, and Arg- β -NA shows Enzyme II activity with a low contribution from the neighbouring Enzyme I. Arg- β -NA was chosen for Enzyme III and Leu- β -NA for Enzyme IV.

Enzyme I was slightly activated by cysteine and EDTA. A marked inhibition was observed in the presence of the metal ions, Ag, Cd, Cu, Hg, Ni, Pb, Zn

and p-CMB. Some other metal ions, including Al, Co, Fe and Sb, were also inhibitory. Enzyme II showed a marked activation by the chelating agents EDTA and 1,10-phenanthroline. The heavy metal ions, Ag, Cd, Cu, Hg and p-CMB, were highly inhibitory. Other metal ions showed a moderate inhibition.

Only a minimal activation was obtained with cysteine for Enzyme III. A dialysed enzyme preparation was used to demonstrate a marked activation by Cl, Br, F and I at 0.1 to 0.2 M concentration. The heavy metal ions (Ag, Cd,

TEXT-FIG. 3. Fractionation of nuclear, mitochondrial and microsomal fractions of the rat testicular tissue by continuous sucrose gradient centrifugation: ○, Arg-β-NA; ■, Val-β-NA; ▲, Ala-β-NA.

Cu, Hg, p-CMB) were highly suppressive, but also the chelating agents (EDTA, α,α' -dipyridyl and 1,10-phenanthroline) and many divalent metal ions were inhibitory to this enzyme.

Enzyme IV was markedly activated by cysteine. This activation was, however, dependent on the time of storage of the enzyme preparation. Within a few days, the enzyme was totally inactivated, but could be reactivated by

FIG. 1. Electron micrograph of Fraction 9 of the continuous sucrose gradient. $\times 30,000$.

FIG. 2. Electron micrograph of Fraction 15 of the continuous sucrose gradient. $\times 30,000$.

(Facing p. 40)

cysteine. Metal ions like Ag, Al, Cd, Cu, Co, Hg, Ni, Pb, Zn and p-CMB were inhibitory to Enzyme IV.

Sucrose gradient fractionation

Val- β -NA, Ala- β -NA and Arg- β -NA were used as substrates in the demonstration of the hydrolytic activities in fractions of sucrose gradient centrifugation of nuclear, mitochondrial(-lysosomal) and microsomal fractions (Text-fig. 3). The nuclear preparation gave only weak activities with all these substrates. In the bottom fractions of the gradient, the nuclear pellet was sedimented and possibly contained cytoplasmic tags which gave low activities with these substrates.

The mitochondrial(-lysosomal) pellet gave a strong area of hydrolysis with all substrates tested in Fractions 5 to 12. The highest activity was obtained with Arg- β -NA as substrate, followed by Val- β -NA and Ala- β -NA. The microsomal preparation showed a low hydrolysis with Val- β -NA in the same fractions, whereas Arg- β -NA disclosed another activity in Fractions 12 to 18.

These studies seemed to indicate that Enzyme I is possibly entirely present in particles of the mitochondrial-lysosomal fraction, while Enzyme II may be partially in the microsomal preparation.

Electron microscopic control

Electron microscope examination of a sample from Fraction 9 of the continuous sucrose gradient fractionation of the mitochondrial-lysosomal preparation showed large amounts of mitochondria, membranous material (Pl. 1, Fig. 1) and occasional lysosomal bodies. Enzyme I may reside in any of these subcellular structures.

A sample from Fraction 15 of the continuous gradient fractionation of the microsomal preparation contained free ribosomes, small vesicles and membranous material, possibly derived from the smooth endoplasmic reticulum (Pl. 1, Fig. 2).

DISCUSSION

The present study revealed four different enzyme activities hydrolysing aminoacyl naphthylamides in the rat testicular tissue. It was obvious that they have distinct differences in substrate specificity, compartmental distribution and subcellular localization. Two of the enzymes (III and IV) were highly soluble and may contribute to the diffuse reaction in the conventional histochemical procedures.

Enzyme III appeared to have characteristics similar to those described for aminopeptidase B in many mammalian tissues with substrate specificity limited to arginyl and lysyl derivatives (Hopsu *et al.*, 1966a, b, c). It showed a strong activation by halide ions as reported previously (Hopsu *et al.*, 1966c). This soluble enzyme was most active in the tubular tissue, but its possible presence in isolated Leydig cells remains to be studied. Due to its solubility, histochemical localization has also been unsuccessful in other tissues. A possible

physiological function in connection with spermatogenic maturation could be anticipated.

Enzyme IV, the other soluble enzyme, appeared to be SH-dependent and was readily inhibited by heavy metal ions and SH-reagents. A high activity of this enzyme could be detected within the seminiferous tubules. Similar enzymes have also been found in other tissues including the pituitary (Vanha-Perttula & Hopsu, 1965; Ellis & Perry, 1966; Vanha-Perttula, 1969), the ovary (Vanha-Perttula & Hopsu, 1966) and the kidney (Vanha-Perttula *et al.*, 1966). These findings indicate that Enzyme IV cannot be a specific testicular enzyme. The final proof of identity with enzymes from other tissues could be effected by utilizing immunological methods with antiserum to a purified enzyme preparation.

Enzyme I was bound to heavy subcellular particles. Electron microscopic control, however, did not clarify the actual site of the particles. Mitochondria, lysosomes or membranous material are all possible sites. Previous studies with other tissues have indicated that mitochondria contain aminopeptidases which hydrolyse aminoacyl naphthylamides and it has been suggested that these enzymes participate in the turnover process of the membranous material (Marks, D'Monte, Bellman & Lajtha, 1970). On the basis of histochemical studies, naphthylamidases have also been connected with the lysosomal bodies (Niemi & Sylvén, 1969; Chayen, Bitensky & Poulter, 1970), but the evidence for this theory is vague and has been criticized (Barrett & Poole, 1969; McDonald, Zeitman & Ellis, 1970). Microvilli in intestinal brush borders (Holt & Miller, 1962) and the plasma membranes of liver and hepatoma cells have also been implicated as possible sites of these enzymes (Emmelot, Visser & Benedetti, 1968).

Enzyme I showed a high specificity for Met-, Ile- and Val- β -NA, resembling an enzyme which has been demonstrated in 2-day-old wound tissue (Mäkinen & Raekallio, 1967, 1969). Both enzymes were also slightly activated by cysteine, inhibited by heavy metal ions (Mäkinen & Raekallio, 1968) and had a pH optimum near neutrality (Mäkinen & Raekallio, 1969). Normal skin or blood does not have such an enzyme and it has been suggested that it is induced during the healing of the tissue injury (Mäkinen & Raekallio, 1969). Since the wound enzyme appears to be identical with that of the testicular tissue, its specificity in tissue regeneration requires further clarification. The evidence from the present study indicates that Enzyme I may be mainly localized within the interstitial tissue, but a weak activity peak in the tubular fraction indicated that the tubular tissue—either the seminiferous epithelium or the Sertoli cells—may also contain this activity. In theory, Enzyme I could be demonstrated in unfixed tissue sections as particulate enzyme. Studies with wound tissue have indicated, however, that a similar wound enzyme cannot be demonstrated by the normal diazo-coupling reaction. One reason may be the SH-activation of the enzyme, which makes it vulnerable to the diazo reagents in the histochemical reaction. Unequivocal proof of a Leydig cell origin for this enzyme can only be shown using isolated Leydig cells.

Enzyme II was localized both in the mitochondrial-lysosomal and microsomal fractions and hydrolysed a wide variety of substrates. The similarity of

this enzyme to those demonstrated in the rat liver and kidney (Mahadevan & Tappel, 1967) and its assumed lysosomal origin remain to be established. In the testicular tissue, some of this activity was also present in the microsomal fractions, which contained smooth membranous material, but no lysosomes or mitochondria. This enzyme appeared to be localized exclusively within the interstitial compartment, since no activity was present on fractionation of the tubular tissue.

The overlapping of substrate specificity interferes with the histochemical demonstration of any one of these four enzymes and also makes its biochemical quantification difficult and biased by the hydrolytic activity of the other enzymes. The present results, however, do suggest some preferential conditions for the quantification of each enzyme. For Enzyme I activity, Val- β -NA or Ile- β -NA are the preferred substrates due to the low contribution of the other enzymes in their hydrolysis. Ala-, His- or Thr- β -NA could be selected for use with Enzyme II, especially with EDTA as an activator. Arg- β -NA or Lys- β -NA can be chosen as substrates for Enzyme III with 0.2 M-NaCl as activator, but Enzymes II and IV will always contribute to the total activity to an undetermined degree. The activity of Enzyme IV is the most difficult to quantify. Its high pH optimum, near pH 8, and activation with cysteine are factors which can be utilized to eliminate the contribution of Enzymes I and II in the hydrolysis of Leu- β -NA or Ala- β -NA.

ACKNOWLEDGMENTS

This work was aided by grants from The Sigrid Jusélius Foundation, Helsinki, Finland, and The National Research Council for Medical Sciences, Finland.

REFERENCES

- BARRETT, A. J. & POOLE, A. R. (1969) Unsuitability of leucine naphthylamide for the histochemical demonstration of lysosomal proteolytic activity. *Nature, Lond.* **224**, 279.
- CHAYEN, J., BITENSKY, L. & POULTER, L. W. (1970) Histochemistry of some lysosomal "proteolytic" enzymes of human rheumatoid synovia. *Nature, Lond.* **225**, 1050.
- ELLIS, S. & PERRY, M. (1966) Pituitary arylamidases and peptidases. *J. biol. Chem.* **241**, 3679.
- EMMELOT, P., VISSER, A. & BENEDETTI, E. L. (1968) Studies on plasma membranes. VII. A leucyl- β -naphthylamidase-containing repeating unit on the surface of isolated liver and hepatoma plasma membranes. *Biochim. biophys. Acta*, **150**, 364.
- GLENNER, G. G., McMILLAN, P. J. & FOLK, J. E. (1962) A mammalian peptidase specific for the hydrolysis of N-terminal α -L-glutamyl and aspartyl residues. *Nature, Lond.* **194**, 867.
- HOLT, J. H. & MILLER, D. (1962) The localization of phosphomonoesterase and aminopeptidase in brush borders isolated from intestinal epithelial cells. *Biochim. biophys. Acta*, **58**, 239.
- HOPUSU, V. K., MÄKINEN, K. K. & GLENNER, G. G. (1966a) Purification of a mammalian peptidase selective for N-terminal arginine and lysine residues: aminopeptidase B. *Archs Biochem. Biophys.* **114**, 557.
- HOPUSU, V. K., MÄKINEN, K. K. & GLENNER, G. G. (1966b) A peptidase (aminopeptidase B) from cat and guinea pig liver selective for N-terminal arginine and lysine residues. I. Purification and substrate specificity. *Acta chem. scand.* **20**, 1225.
- HOPUSU, V. K., MÄKINEN, K. K. & GLENNER, G. G. (1966c) A peptidase (aminopeptidase B) from cat and guinea pig liver selective for N-terminal arginine and lysine residues. II. Modifier characteristics and kinetic studies. *Acta chem. scand.* **20**, 1231.
- HOPUSU-HAVU, V. K., MÄKINEN, K. K. & GLENNER, G. G. (1966) Formation of bradykinin from kallidin-10 by aminopeptidase B. *Nature, Lond.* **212**, 1271.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

- McDONALD, J. K., ZEITMAN, B. B. & ELLIS, S. (1970) Leucine naphthylamide: an inappropriate substrate for the histochemical detection of cathepsin B and B'. *Nature, Lond.* **225**, 1048.
- MAHADEVAN, S. & TAPPEL, A. L. (1967) Arylamidases of rat liver and kidney. *J. biol. Chem.* **242**, 2369.
- MÄKINEN, P. L. & RAEKALLIO, J. (1967) Studies on the formation of amino-peptidase-like enzymes in regenerating wound tissue. *Acta chem. scand.* **21**, 761.
- MÄKINEN, P. L. & RAEKALLIO, J. (1968) Fractionation and characterization of arylamidases in regenerating wound tissue. *Acta chem. scand.* **22**, 597.
- MÄKINEN, P. L. & RAEKALLIO, J. (1969) Characterization of four arylaminopeptidases of rat skin. *Enzymologia*, **36**, 93.
- MARKS, N., D'MONTE, B., BELLMAN, C. & LAJTHA, A. (1970) Protein metabolism in cerebral mitochondria. I. Hydrolytic enzymes and amino acid incorporation into mitochondrial membranes. *Brain Res., Amst.* **18**, 309.
- NAGATSU, I., NAGATSU, T., YAMAMOTO, T., GLENNER, G. G. & MEHL, J. W. (1970) Purification of aminopeptidase A in human serum and degradation of angiotensin II by the purified enzyme. *Biochim. biophys. Acta*, **198**, 255.
- NIEMI, M. & SYLVÉN, B. (1969) The naphthylamidase reaction as a diagnostic tool for the demonstration of cellular injury and autophagy. *Histochemie*, **18**, 40.
- TAKENAKA, M. (1964) A new method for the determination of leucine aminopeptidase in serum (use of p-dimethylaminobenzaldehyde for coloration). *Bull. Yamaguchi med. Sch.* **11**, 57.
- VANHA-PERTTULA, T. (1969) Aminoacyl and dipeptidyl arylamidases (aminopeptidases) of the pituitary gland as related to function. *Endocrinology*, **85**, 1062.
- VANHA-PERTTULA, T. P. J. & HOPUS, V. K. (1965) Esterolytic and proteolytic enzymes of the rat adenohypophysis. II. Chromatographic fractionation and characterization of enzyme activities hydrolysing leucyl- β -naphthylamide. *Annls Med. exp. Biol. Fenn.* **43**, 32.
- VANHA-PERTTULA, T. P. J. & HOPUS, V. K. (1966) The ovarian enzymes hydrolysing L-leucyl- and DL-alanyl- β -naphthylamide. *Histochemie*, **6**, 34.
- VANHA-PERTTULA, T., HOPUS, V. K. & GLENNER, G. G. (1966) Enzymes in hog kidney hydrolysing amino acid naphthylamides. *J. Histochem. Cytochem.* **14**, 314.