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COMMENTARY

ARE THERE NEUROPEPTIDE-SPECIFIC PEPTIDASES?

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Since the discovery of the enkephalins in 1975 [1], the neuronal distribution of CNS peptides has been mapped in some considerable detail (see, for example, Ref. 2). In contrast, an understanding of the mechanism of synaptic inactivation of the neuropeptides has been slow to develop. Since there are now more than thirty peptides identified in brain, it would be very surprising if separate mechanisms existed for the inactivation of each peptide at the synapse [2]. By analogy with "classical" neurotransmitter systems, inactivation may occur by either extracellular metabolism (as is the case for acetylcholine [3]) or re-uptake followed by intracellular metabolism (as for γ-aminobutyrate [4]). Although the involvement of uptake mechanisms cannot yet be rigorously excluded for neuropeptide inactivation, the consensus of opinion is that the physiological action of neuropeptides is normally terminated by extracellular metabolism [2, 5, 6]. This review will therefore be concerned with the membrane peptidases that are strategically located at the cell surface to carry out this process. Intracellular peptidases, including those in the cytosol (e.g. post-proline endopeptidase, EC 3.4.21.26) and those in lysosomes (e.g. the cathepsins), will not be considered further. Peptidases are generally classified as either exopeptidases (removing one or two residues from either the N- or the C-terminus) or endopeptidases (hydrolysing at internal sites in peptide chains). Where the substrates are relatively small peptides, as is the case for many of the neuropeptides, these distinctions can become blurred. However, since many biologically active peptides have blocked N- and C-termini, e.g. gastrin, LH-RH, α-MSH, caerulein, and bombesin, the action of an endopeptidase will commonly be required to initiate hydrolysis of a given peptide.

Several reviews of neuropeptide metabolism have appeared recently [7–11]. These reviews have generally focused on one or more specific peptides and compared their sites of inactivation. Such an approach can often imply the existence of highly specific peptidases (e.g. "enkephalinase") and can obscure the role that well-characterized peptidases may play in this process. The present review will adopt a different standpoint. We will first describe the enzymology of established plasma membrane peptidases and then try to relate this information to the potential neuropeptide substrates for these enzymes. The approaches that have been used for the development of selective peptidase inhibitors will also be highlighted. The membrane peptidases best characterized in molecular terms are undoubtedly those located in the brush borders of kidney and intestine [12]. At first sight these enzymes may not seem appropriate to a discussion of the metabolism of peptides at the synapse, but it is becoming clear that the plasma membranes of diverse cell types express a battery of common peptidases that subserve different functions in different tissues.

Kidney and intestinal microvillar peptidases

The peptidases that have been characterized in the kidney microvillar membrane are listed in Table 1. These enzymes, which happen to be highly concentrated in the renal brush border, are not unique to the proximal tubule cell. A comparable assembly of peptidases is present in intestinal microvilli. The complementary nature of the peptidases provides an efficient mechanism for digesting any small peptide to its constituent amino acids. The precise functions of the kidney peptidases remain a mystery. Although it is known that they degrade many peptide hormones present in the glomerular filtrate, it is not clear why this process should be mandatory for hormones already sequestered from the circulation [14]. Enzymes with similar properties have now been identified in membranes from various tissues, including brain, and several have been shown to be identical to the kidney peptidases by immunological criteria [15–19]. Thus, the microvillar enzymes can provide a useful model for the study of membrane peptidases from other tissues. Moreover, after enzyme identity between tissues has been established, the abundant kidney peptidases can be more conveniently used for studies on the specificity of peptide hydrolysis.

The microvillar peptidases listed in Table 1 are all integral membrane glycoproteins that share a number of structural similarities. They possess large subunits (M_r usually > 80,000) and exist as dimers of identical subunits in the membrane (exceptions being aminopeptidases A and N and endopeptidase-24.11 in the rabbit, where they are monomeric; see Ref. 12). The bulk of the protein is hydrophilic and glycosylated, with only a small hydrophobic domain (<5% of the mass of the protein) apparently near the N-terminus comprising the transmembrane portion. In all cases, the active site is located on the hydrophilic domain facing the extracellular space.

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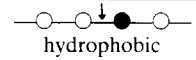
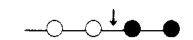
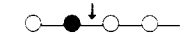
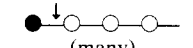
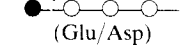
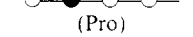
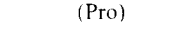
Table 1. Kidney and intestinal microvillar peptidases*

Enzyme	Active site	Subunit M_r (pig)	Specificity	Usual assay substrates	Presence in CNS
Endopeptidase-24.11	Zn^{2+}	90	 hydrophobic	[^{125}I]Iodoinsulin B chain; [D-Ala^2,Leu^5]enkephalin; glutaryl-Ala-Ala-Phe-X	Yes
Peptidyl dipeptidase A	Zn^{2+}	195	 (many)	Hip-His-Leu	Yes
Dipeptidyl peptidase IV	Serine	130	 (Pro)	Gly-Pro-X	Yes
Aminopeptidase N	Zn^{2+}	160	 (many)	Ala-X; Leu-X	?
Aminopeptidase A	Ca^{2+}	170	 (Glu/Asp)	Asp-X; Glu-X	?
Aminopeptidase P	?	?	 (Pro)	Gly-Pro-HyPro	?
Proline carboxypeptidase	?	?	 (Pro)	Z-Pro-Met	?

* X may be 2-naphthylamide, 4-nitroanilide or 7-amido-4-methylcoumarin. Hip = hippuryl (benzoylglycyl); HyPro = hydroxyproline. The specificity requirements for the amino acid residue (●) are indicated in parentheses. In addition to the above enzymes, synaptic membranes contain a thyroliberin hydrolyzing pyroglutamate aminopeptidase [13].

They are therefore all "ectoenzymes" in the terminology of Engelhardt [20]. Most of the peptidases (endopeptidase-24.11 is one exception) can be released from the membrane in an active hydrophilic form by proteinase treatment [12]. The proteolysed form of the enzyme lacks the hydrophobic membrane anchor. Since there is a common mechanism for the biosynthesis and assembly of membrane proteins, it is likely that the structure and orientation of brain membrane peptidases are similar to those for the microvillar enzymes, although some tissue differences in post-translational processing may occur. The mode of biosynthesis and assembly of microvillar proteins has been described elsewhere [21] and will not be discussed further here.

Brain membrane peptidases

In this review we are concerned exclusively with the peptidases of the plasma membrane. It is pertinent, therefore, to consider criteria for adjudging a particular enzyme to be membrane-associated. The term "membrane peptidase" has been used loosely in the literature, particularly in relation to the location of brain peptidases. An integral membrane protein is, by definition, amphipathic and, when solubilized by detergent treatment, becomes associated with a micelle of detergent. In the absence of detergent, such proteins will form high molecular weight aggregates (protein micelles) and may also lose activity. The need for detergents can sometimes be avoided by releasing the protein from the membrane in a hydrophilic form by limited proteolysis (see above). However, integral proteins are not removed from the membrane merely by washing with solutions of low or high ionic strength, a treatment that serves only to remove the bulk of any adherent cytosolic proteins as well as peripheral membrane proteins that are associated electrostatically. It is therefore useful to monitor in parallel the release of cytosolic markers such as lactate dehydrogenase from a crude membrane preparation. Other criteria for identifying amphipathic proteins include charge-shift electrophoresis [22] and hydrophobic chromatography or immunoelectrophoresis [23]. The peptidases discussed below all exist as integral proteins of the plasma membrane and are widely distributed in mammalian tissues.

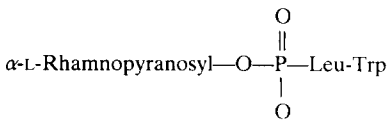
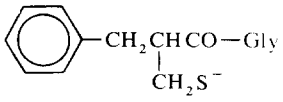
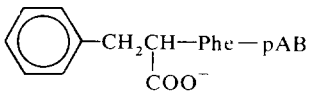
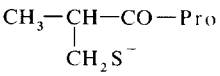
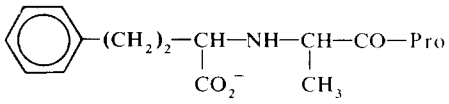
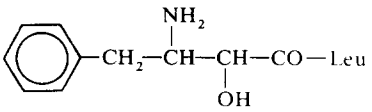
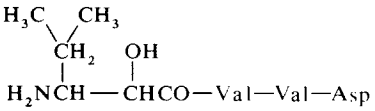
Endopeptidase-24.11 ("enkephalinase")

The main focus of attention in the field of neuropeptide metabolism has been the enzymes involved in the physiological inactivation of the enkephalins, since selective inhibition of this metabolic process could lead to the design of new analgesics. Major importance has been attached to the membrane enzyme hydrolysing the Gly^3-Phe^4 bond of enkephalin, an activity that has been termed "enkephalinase" [7, 24], implying an exclusive role for this peptidase in the CNS. This activity is neither brain- nor neuropeptide-specific. It has now been established unequivocally [17–19], that brain "enkephalinase" is identical with endopeptidase-24.11 (EC 3.4.24.11), an enzyme first purified from kidney microvilli, where it is particularly abundant [25, 26]. Similarity of the kidney and brain activities was indicated by their sensitivity to the inhibitors thiorphan and phosphoramidon (see Table 2) [27]. Proof of identity has come from immunological criteria [17–19], particularly the use of a monoclonal antibody to the kidney endopeptidase for purification of the brain enzyme by immunoadsorbent chromatography [18]. The physiological function of endopeptidase-24.11 must be viewed in the light of its wide distribution in mammalian tissues [28], many of which do not contain endogenous enkephalin. In view of its broad specificity, we have proposed that endopeptidase-24.11 plays a general role in the hydrolysis and inactivation of biologically active peptides at cell surfaces [17].

Kidney, intestine and brain endopeptidase-24.11 are all glycoproteins, which appear identical in amino acid composition but differ somewhat in their pattern of glycosylation [18]. It is likely that the polypeptide chain ($M_r = 77{,}000$) is the same gene product in these different cell types. Studies from a number of laboratories, including our own, allow some general comments to be made on the specificity of the enzyme. The peptide bonds hydrolysed are those involving the amino groups of hydrophobic residues (Phe, Leu, Ile, Val, Tyr, Trp). However, other residues in the vicinity of the scissile bond can markedly affect the efficiency of substrate hydrolysis producing variations in k_{cat}/K_m values of more than two orders of magnitude with natural peptides (Fig. 1). As yet, no simple predictive rules for "good" or "bad" substrates have emerged. The observation that enkephalinamide was a relatively poor substrate [7, 29] led Schwartz and colleagues to suggest that the enzyme functioned as a peptidyl dipeptidase requiring a free α-carboxyl group at the C-terminus for efficient hydrolysis to occur. This is by no means the case since enkephalins containing C-terminal extensions (e.g. [Met^5]enkephalin-Arg^6-Phe^7) are hydrolysed at the Gly^3-Phe^4 bond at least as efficiently as the parent peptide. Furthermore, substance P, which possesses a C-terminal amide group, is one of the best substrates for the enzyme ($k_{cat}/K_m = 3.6$ relative to [Leu^5]enkephalin) [30]. Cholecystokinin (CCK), neurotensin, bradykinin, insulin B-chain and other peptides are also hydrolysed efficiently. In the case of CCK, the enzyme proved to be capable of releasing the C-terminal phenylalanylamide from the peptide [31]. The sites of hydrolysis confirm that the enzyme is correctly identified as an endopeptidase. Further structure–activity studies will be required to explain the anomalous behaviour of enkephalinamide. The hydrolyses of enkephalin, substance P and CCK by synaptic membranes are potently inhibited by phosphoramidon and thiorphan, suggesting that endopeptidase-24.11 may play a significant role in terminating the biological activity of each of these peptides [17, 31, 32]. Furthermore, thiorphan has been shown to potentiate enkephalin-induced analgesia *in vivo* [33]. The hydrolysis of certain other peptides (e.g. LH-RH and neurotensin) by synaptic membranes is far less sensitive to inhibition by thiorphan or phosphoramidon, suggesting that other peptidases are of importance in degrading these peptides (unpublished observations).

Specificity and inhibitor studies *in vitro* can provide only a guide as to the likely physiological substrates for a given peptidase. It is essential to com-

Table 2. Characteristic inhibitors of membrane peptidases

Enzyme	Inhibitors	Structure*	References
Endopeptidase-24.11	Phosphoramidon		[27, 96]
	Thiorphan		[33]
	CPAB		[94]
Peptidyl dipeptidase A	Captopril		[48]
	MK422 (enalapril diacid)		[91]
Aminopeptidases	Bestatin		[97, 98]
	Amastatin		[98, 99]

* pAB = *p*-aminobenzoate; and CPAB = *N*-[1(*R,S*)-carboxyl-2-phenylethyl]-Phe-*p*-aminobenzoate.

plement these studies with a detailed immunocytochemical approach in order to show whether each peptidase is located in the synaptic membrane and with the appropriate orientation for it to have a physiological role in hydrolysing neuropeptides released at that site. Although there are detailed studies on the localization of neuropeptides in the CNS (see, for example, Ref. 2), relatively little is known of the distribution of peptidases. Endopeptidase-24.11 is relatively abundant in striatum and spinal cord, and its overall distribution in brain is not inconsistent with a role in the metabolism of enkephalin or substance P ([34], and unpublished observations). The enzyme is enriched in synaptosomal preparations [35] but has also been reported to be present on glial cell membranes [36]. More precise immunocytochemical data on the localization of the endopeptidase in the nervous system have not yet been reported.

Peptidyl dipeptidase A (angiotensin converting enzyme, ACE)

Peptidyl dipeptidase A (EC 3.4.15.1) is also widely distributed in mammalian tissues. Its major location is considered to be the lung, where it catalyses the inactivation of bradykinin and the hydrolysis of angiotensin I to yield the vasoactive peptide angiotensin II [37]. Inhibitors of peptidyl dipeptidase A such as captopril have therefore found application as anti-hypertensive agents [38]. Brain tissue also contains the components of the renin-angiotensin system, including peptidyl dipeptidase A, which is enriched in synaptosomes [39]. It is likely that the enzyme plays a far wider role in the metabolism of biologically active peptides than is implied by its trivial name, angiotensin converting enzyme. The primary specificity of the enzyme is to release dipeptide fragments sequentially from the C-terminus of an oligopeptide (see Table 1). For example, the

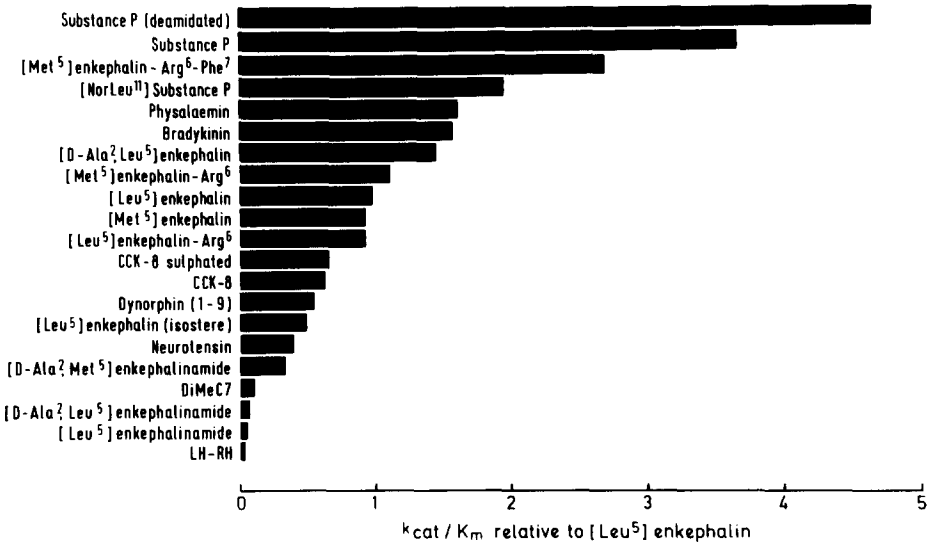


Fig. 1. Kinetics of hydrolysis of neuropeptides and their analogues by endopeptidase-24.11. The data are expressed as k_{cat}/K_m relative to [Leu^5]enkephalin = 1.0.

enzyme releases His-Leu from the C-terminus of angiotensin, Phe-Arg from bradykinin and can release Phe-Leu from the C-terminus of [Leu^5]enkephalin *in vitro* [40]. The hydrolysis of the Gly^3-Phe^4 bond of enkephalin *in vivo* appears to be primarily a function of endopeptidase-24.11 (see above), although it is possible that peptidyl dipeptidase A plays a role in the metabolism of enkephalin precursors [41]. An unexpected feature of peptidyl dipeptidase A is the recent demonstration that it can hydrolyse the Phe^8-Gly^9 bond of the neuropeptide substance P [42, 43]. Since this peptide is amidated at the C-terminus, the enzyme can, therefore, in certain circumstances, display endopeptidase activity. Clearly, both the specificity and the physiological function of peptidyl dipeptidase A need re-examination since there is some evidence that the enzyme may contribute to the metabolism of substance P *in vivo* in some tissues [43]. The enzyme may also play a role in the metabolism of neurotensin [44], but its ability to hydrolyse other neuropeptides has received little attention.

Like endopeptidase-24.11, peptidyl dipeptidase A is a glycoprotein containing a Zn^{2+} atom at the active site. Chemical modification studies have also revealed the presence of reactive glutamyl, arginyl and tyrosyl residues at the active site [45, 46]. Substrate specificity studies indicate that the enzyme shows some preference for those peptides containing an aromatic residue in the P_1 position adjacent to the scissile bond [47]. However, the enzyme is far less selective than endopeptidase-24.11. For many, but not all, substrates, maximal rates of hydrolysis occur in the presence of chloride ions, which affect the K_m for substrate binding. A series of peptide derivatives containing a prolyl residue at the C-terminus (which hinders hydrolysis) has been investigated and has led to several potent inhibitors of the enzyme (see below). From the detailed studies that have been carried out, models have been proposed for the active site region of peptidyl dipeptidase A [48] and have been used as the basis for the design of more potent and selective inhibitors (see below).

Some clues to the possible physiological roles of peptidyl dipeptidase A in the brain may come from a knowledge of its anatomical and cellular localization. Immunocytochemical studies have localized peptidyl dipeptidase A in capillary endothelial cells throughout the brain, and particularly in the brush border of the choroid plexus [49, 50]. This localization is different from that of angiotensin I and of endopeptidase-24.11 in the brain. There also appears to be some detectable peptidyl dipeptidase A activity in the neuronal striatonigral pathway [51]. The use of [^{3}H]captopril, which binds uniquely and with high affinity to peptidyl dipeptidase A has aided in tracing pathways in the brain that contain the enzyme [52].

Aminopeptidases

Mammalian tissues contain an array of cytosolic and membrane-bound aminopeptidases [53]. The best-characterized microvillar aminopeptidase is aminopeptidase N, EC 3.4.11.2 (formerly designated aminopeptidase M). This is a membrane glycoprotein of subunit M_r approx. 160,000 [12]. It is also a metalloenzyme containing Zn^{2+} at the active site. Aminopeptidase N is distinct in structure and differs somewhat in substrate specificity from the cytosolic enzyme leucine aminopeptidase (EC 3.4.11.1) [54]. The membrane enzyme shows a broad side-chain specificity with alanyl residues being hydrolysed the most rapidly. Phenylalanine, tyrosine, leucine and arginine derivatives are also hydrolysed at substantial rates. Much lower rates of hydrolysis are seen with glutamate, aspartate and proline [55]. Other aminopeptidases in the microvillar membrane include aminopeptidase A (aspartate aminopeptidase, EC 3.4.11.7) with specificity for N-terminal Glu and Asp residues, and aminopeptidase P which will release an N-terminal residue adjacent to a proline. Synaptic membranes contain

an enzyme with some characteristics similar to aminopeptidase N (see below). The occurrence of aminopeptidases P and A in synaptic membranes has not been reported although the release of the N-terminal aspartyl residue of CCK-8 may be attributable to aminopeptidase A [32].

Regulatory peptides that are unprotected at the N-terminus, e.g. the enkephalins, will be susceptible to the action of aminopeptidases. Initial studies on the inactivation of enkephalin by brain homogenates revealed that a major site of hydrolysis was the Tyr^1-Gly^2 bond [56, 57]. Much of this catalytic activity proved to be cytosolic [58], but significant quantities of membrane-bound aminopeptidase activity have also been reported [58–60]. Although endopeptidase-24.11 has been hailed as the "enkephalinase" of opiate synapses [7, 24], the membrane aminopeptidase activity exceeds that of endopeptidase-24.11 in all brain regions examined ([61], and unpublished observations). Thus, it is likely that a combination of both enzymes is involved in the inactivation of the enkephalins. Some support for this contention has come from the observation that inhibition of both endopeptidase-24.11 (with thiorphan) and of aminopeptidase (with bestatin) provides greater potentiation of enkephalin-induced analgesia than is produced by inhibition of either enzyme alone [62]. Since the ratio of aminopeptidase:endopeptidase-24.11 varies greatly between tissues and even between different brain regions, the relative importance of the two activities will show marked regional and tissue differences. Indeed, the inhibition of endopeptidase-24.11 (with thiorphan) or peptidyl dipeptidase A (with captopril) failed to potentiate contractions of the guinea pig ileum induced by [Met^5]lenkephalin [63], and aminopeptidase activity was concluded to be primarily responsible for enkephalin inactivation, at least in the ileum [63]. The role of aminopeptidases in the metabolism of other susceptible neuropeptides has been little investigated, but recent experiments on the hydrolysis of CCK-8 (sulphated) by synaptic membranes show that both a bestatin-sensitive aminopeptidase and endopeptidase-24.11 are involved in its degradation [31, 32].

A number of recent studies have tried to characterize the brain aminopeptidases that hydrolyse the Tyr^1-Gly^2 bond of enkephalin. Most of these investigations have centred on the cytosolic enzymes which have been purified from monkey, rat, human and bovine brain [63–67]. They closely resembled cytosolic aminopeptidase present in other tissues. Of greater physiological interest in terms of enkephalin metabolism are the membrane aminopeptidases. Hersh [68] has reported two membrane enzymes from bovine brain able to cleave the Tyr^1-Gly^2 bond of enkephalin. One of these (designated M2) had many properties in common with cytosolic aminopeptidase (e.g. similar K_m for enkephalin and sensitivity to puromycin). The other enzyme (M1) was distinct in properties from cytosolic aminopeptidase, and had some characteristics in common with aminopeptidase N. However, it was not possible to confirm its identity unambiguously. Hui *et al.* [69] have also purified an "enkephalin aminopeptidase" from rat brain membranes. This enzyme was reported to be a metalloprotein, sensitive to inhibition by bestatin, and exhibited a K_m for enkephalin in the millimolar range. Under the conditions of assay, tyrosine was released from enkephalin but not the subsequent glycine residues. On sodium dodecyl sulphate (SDS)-gel electrophoresis, the rat brain enzyme revealed two subunits of M_r 62,000 and 66,000, which contrasts with the subunit size of pig kidney microvillar aminopeptidase N (M_r = 160,000). However, purification of the solubilized rat brain aminopeptidase was performed in the absence of detergent, apparently without aggregation of the protein [69]. This suggests that a hydrophilic form of the enzyme, perhaps arising by proteolysis, had been purified. There may also be species differences in the subunit size of aminopeptidases, since rat intestinal aminopeptidase N also appears to be smaller than the corresponding pig enzyme [70]. Whether the membrane form of "enkephalin aminopeptidase" is identical with aminopeptidase N is still, therefore, unresolved, and immunological criteria will be needed to confirm identity. Although bestatin has proved useful as an aminopeptidase inhibitor for studies of enkephalin metabolism [62], there is currently no specific inhibitor of the membrane aminopeptidase that approaches the selectivity or potency with which phosphoramidon (K_i = 2 nM), or thiorphan, inhibits endopeptidase-24.11. The development of such aminopeptidase inhibitors would allow a more definitive study of the role of the enzyme in the primary inactivation at the cell surface of peptides possessing free α-amino groups.

Other peptidases

Much less is known of other cell-surface peptidases in the brain. A few, though, deserve consideration. Dipeptidyl peptidase IV (3.4.14.5) is present in synaptic membranes and is capable of hydrolysing artificial substrates such as Gly-Pro-2-naphthylamide to liberate the dipeptide Gly-Pro. The enzyme is widely distributed in mammalian tissues but the richest source of the enzyme is the kidney where it is located in the microvillus membrane [71]. It is a serine enzyme [72] with a subunit M_r of 130,000 (Table 1). A number of studies have dealt with the specificity of the enzyme [72, 73]. In general, Pro or Ala adjacent to the N-terminal residue renders a peptide susceptible to hydrolysis, although Pro is preferred. The enzyme is distinct from, and should not be confused with, post-proline endopeptidase (post-proline cleaving enzyme; EC 3.4.21.26) which is a cytosolic enzyme [11]. Among recognised neuropeptides, two potential substrates for dipeptidyl peptidase IV stand out: substance P (N-terminal sequence, Arg-Pro-Lys-Pro . . .) and bradykinin (Arg-Pro-Pro . . .). Purified dipeptidyl peptidase IV has been shown to hydrolyse substance P *in vitro*, but not bradykinin [73, 74]. The lack of a selective and non-toxic inhibitor of the enzyme makes it difficult to assess whether the enzyme may play a role in substance P metabolism *in vivo*. However, since the biological activity of substance P resides in its C-terminal portion, the action of dipeptidyl peptidase IV alone is not sufficient to inactivate the neuropeptide. Another membrane peptidase that has been implicated in substance P metabolism has been

designated "substance P-degrading enzyme" [75], an endopeptidase that has been purified from human brain. The enzyme demonstrated an unusual specificity for a single peptidase in that it hydrolysed bonds involving the amino group of both phenylalanyl and glycyl residues. Hydrolysis was not inhibited by phosphoramidon nor captopril. The reported properties do not accord with any known cell-surface peptidase, and a recent report suggests that this activity may be mitochondrial in location [76]. Any role in the physiological inactivation of substance P is therefore questionable at present. Indeed, we have observed that the metabolism of substance P by synaptic membranes from human striatum was inhibited by phosphoramidon (R. Matsas, A. J. Kenny and A. J. Turner, unpublished observations), and therefore attributable to endopeptidase-24.11, in agreement with our previous results from pig striatum [17].

Finally among cell-surface enzymes, mention should be made of the apparent ability of acetylcholinesterase to function as a peptidase. Several reports have indicated that highly purified preparations of acetylcholinesterase from different sources can hydrolyse substance P, with hydrolysis principally occurring at the Leu^{10}-$Met^{11}NH_2$ bond [77, 78]. Hydrolysis of the enkephalins and their precursors has also been detected [78, 79] but not of angiotensin II, oxytocin, bombesin or vasopressin. Although acetylcholine inhibited peptide hydrolysis, the peptidase activity was far less sensitive to diisopropylfluorophosphate than the esterase activity [78]. Thus, the evidence that acetylcholinesterase may play a role in the metabolism of peptides is circumstantial, although some support comes from the co-localisation of substance P and acetylcholinesterase in the dorsal horn of the spinal cord, a region that contains little or no acetylcholine or choline acetyltransferase.

Inhibitors of membrane peptidases

The design of potent and selective inhibitors of cell-surface metallopeptidases owes much to the studies on the structures and mechanism of action of the zinc-containing enzymes, carboxypeptidase A and thermolysin, which have been used as models for peptidyl dipeptidase A and endopeptidase-24.11 respectively. In addition, a number of naturally occurring compounds which inhibit these enzymes has also aided the synthesis of new inhibitors. The snake venom peptide, Glp-Lys-Trp-Ala-Pro (bradykinin potentiating factor 5a), is the most potent of a series of such peptides at inhibiting peptidyl dipeptidase A, although it is susceptible to hydrolysis by the enzyme [80]. Another venom peptide, SQ20881 (Glp-Trp-Pro-Arg-Pro-Glu-Ile-Pro-Pro), is resistant to hydrolysis and is an effective antihypertensive agent, but with the disadvantage that it is not orally active [81, 82]. The C-terminal tripeptide sequence Trp-Ala-Pro appears to be most favourable for binding to the active site of peptidyl dipeptidase A [83, 84]. The C-terminal prolyl residue is not favourable for interaction with endopeptidase-24.11.

Structural studies of carboxypeptidase A have revealed that a basic group (Arg^{145}) at the active site forms an ionic bond with the terminal carboxyl group of the substrate [85]. An adjacent hydrophobic pocket is responsible for the specificity of carboxypeptidase A towards substrates containing a C-terminal aromatic amino acid. The zinc atom at the active site participates directly in the catalytic mechanism. A compound that interacts stereospecifically with each of these sites of carboxypeptidase A, e.g. D-benzylsuccinate, produces particularly potent inhibition [86]. The active site requirements of both peptidyl dipeptidase A and endopeptidase-24.11 show some similarities to carboxypeptidase A although, in the case of peptidyl dipeptidase A, the requirement for a hydrophobic pocket is not essential. On this basis, Ondetti *et al.* designed the first of the orally active peptidyl dipeptidase A inhibitors, captopril (D-3-mercapto-2-methylpropanoyl-L-proline) which is structurally analogous to the dipeptide Ala-Pro, but is stable to hydrolysis by peptidases [48]. The essential features of captopril that produce potent inhibition (K_i = 2 nM) are the presence of a thiol group as a zinc-ion ligand and a prolyl residue at the C-terminus. Captopril has found wide use as an anti-hypertensive agent [38, 87] although not without some side-effects [88], which has led to an intensive search for new classes of peptidyl dipeptidase A inhibitors *in vivo* (see, for example, Ref. 89 and 90), the most promising of which appears to be enalapril (*N*-((*S*) -1 -(ethoxycarbonyl)-3-phenylpropyl)-L-alanyl-L-proline maleate), a non-sulphydryl containing inhibitor [91].

Similar strategies have been used for the design of inhibitors of endopeptidase-24.11 [92–95]. A natural product from *Streptomyces tanashiensis*, phosphoramidon, inhibits thermolysin and was the first compound reported to inhibit specifically endopeptidase-24.11 [96]. However, the increasing interest in this enzyme over the last few years has led several laboratories to design new compounds. Some of these are shown in Table 2. The impetus for this research derives, at least in part, from the possibility that a selective inhibitor of endopeptidase-24.11 might have analgesic properties and lead to a useful therapeutic compound. The conceptual approach that led to the design of captopril as an inhibitor of peptidyl dipeptidase A [48] was the basis on which the thiol compound, thiorphan, was synthesized [33]. This was later modified by reversing the —CO—NH— groups to give retrothiorphan [100], a compound that would thus resist attack by carboxypeptidases. Other laboratories [94] have introduced C-terminal substituents, a feature that may likewise increase the biological stability of the inhibitor. However, all the effective compounds have two common features: (a) a group capable of coordinating with the active-site zinc ion, and (b) a hydrophobic amino acid residue at the P'_1 position. Since peptidyl dipeptidase A is also a zinc metalloenzyme with a wide specificity, it is not surprising that some of the compounds have overlapping inhibitory activities. The IC_{50} values for the hydrolysis of [D-Ala^2,Leu]enkephalin by endopeptidase-24.11 and Hip-His-Leu by peptidyl dipeptidase A are shown in Fig. 2 (from Ref. 30). Interestingly, the natural product, phosphoramidon, remains one of the most potent inhibitors of the endopeptidase, but it is not wholly specific [30]. Differences in the structural requirements of inhibi-

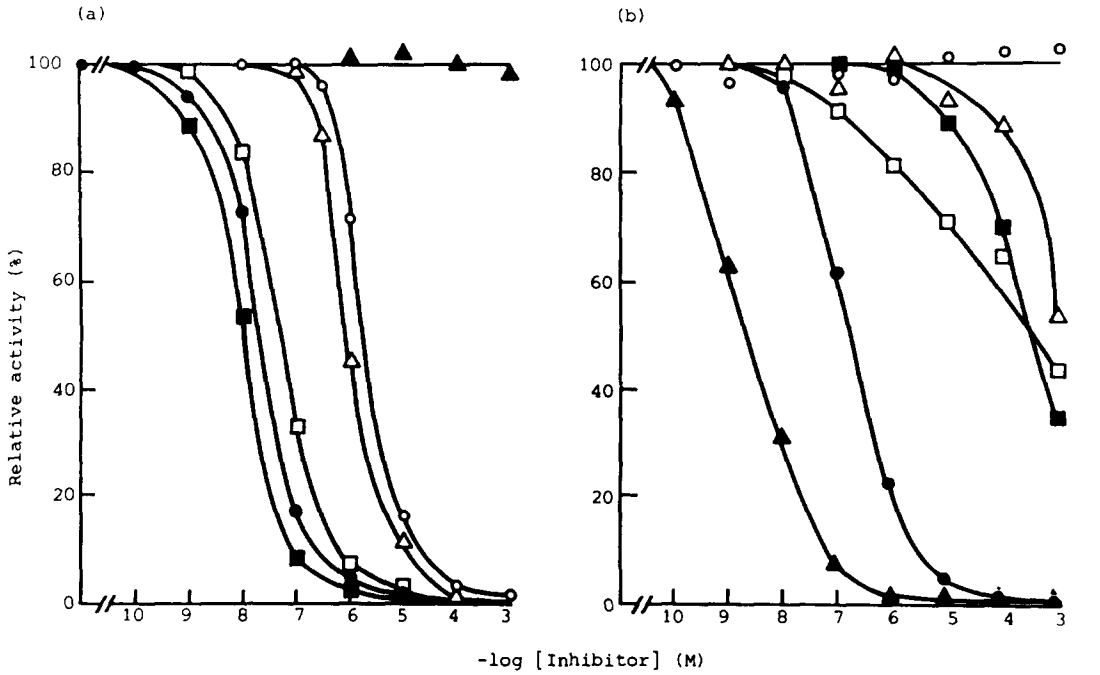


Fig. 2. Inhibition of endopeptidase-24.11 and peptidyl dipeptidase A. (a) Inhibition of endopeptidase-24.11. (b) Inhibition of peptidyldipeptidase A. The inhibitors used were: (○) CPAB; (△) UK55076; (□) *retro*-thiorphan; (●) thiorphan; (▲) MK422; and (■) phosphoramidon. The effectors were present in the incubation mixture at the concentration shown and were added before the substrate, but no preincubation was used. Endopeptidase-24.11 was assayed with [D-Ala^2,Leu^5]enkephalin and peptidyl dipeptidase A with Hip-His-Leu as substrate (taken from Ref. 30).

tors for selective interaction with endopeptidase-24.11 or peptidyl dipeptidase A are currently being defined (see, for example, Refs. 30 and 95). The therapeutic potential of selective endopeptidase inhibitors remains to be evaluated.

Although a number of neuropeptides (e.g. enkephalin, CCK) are susceptible to the action of aminopeptidases, the development of novel inhibitors of these enzymes has attracted less interest than for the other peptidases described. Puromycin has commonly been used as a general inhibitor of aminopeptidase activity in studies of neuropeptide metabolism [7, 101], but is neither particularly potent nor specific. Two natural products, bestatin and amastatin, are more potent aminopeptidase inhibitors [97] and may prove useful as a starting point for the synthesis of more effective compounds. Bestatin was originally characterized as an inhibitor of aminopeptidase B, but also inhibits other aminopeptidases and is particularly effective as an inhibitor of kidney cytosolic leucine aminopeptidase (EC 3.4.11.1) [98, 102]. Amastatin was isolated as an inhibitor of aminopeptidase A (EC 3.4.11.7), but exhibits an overlapping specificity against other aminopeptidases [99]. Bestatin (in combination with thiorphan) has been shown to protect endogenous enkephalin released from brain slices against metabolism by extracellular peptidases [62]. However, such peptide release experiments need to be interpreted with caution [103]. A novel approach to inhibitor design has produced kelatorphan, a compound that inhibits all the enzymes of enkephalin metabolism with relatively high potency [104] and which may therefore be useful in potentiating the physiological actions of the enkephalins.

Conclusions

Although peptidases of high specificity do exist, such a close relationship between peptidase and substrate is unusual. We have suggested that a limited number of cell-surface peptidases mediate a wide range of functions and that their cellular and subcellular localization, rather than peptide specificity, defines their roles at different tissue sites. Although studies of neuropeptide metabolism are still in their infancy, this hypothesis certainly appears to be vindicated for the inactivation of the enkephalins, substance P and cholecystokinin. However, the localization of many of the peptidases within the nervous system remains to be defined and adequate techniques need to be developed for assessing the physiological role of identified peptidases in the metabolism of individual neuropeptides.

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Note added in proof: It has been reported recently that approximately 10% of brain membrane aminopeptidase activity is immunologically similar to kidney microvillar aminopeptidase N [Gros *et al.*, *Biochem. Soc. Trans.* **13**, 47 (1985)].

REFERENCES

1. J. Hughes, T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan and H. R. Morris, *Nature, Lond.* **158**, 577 (1975).

2. D. T. Krieger, *Science* **222**, 975 (1983).
3. V. P. Whittaker, M. J. Dowdall and A. F. Boyne, in *Neurotransmitters and Metabolic Regulation* (Ed. R. M. S. Smellie). *Biochem. Soc. Symp.* **36**, 49 (1972).
4. A. J. Turner and S. R. Whittle, *Biochem. J.* **209**, 29 (1983).
5. L. L. Iversen, T. Jessell and I. Kanazawa, *Nature, Lond.* **264**, 82 (1976).
6. T. Segawa, Y. Nakata, H. Yajima and K. Kitagawa, *Jap. J. Pharmac.* **27**, 573 (1977).
7. J.-C. Schwartz, B. Malfroy and S. de la Baume, *Life Sci.* **29**, 1715 (1981).
8. J-C. Schwartz, *Trends Neurosci.* **6**, 45 (1983).
9. E. C. Griffiths and J. R. McDermott, *Molec. cell. Endocr.* **33**, 1 (1983).
10. L. B. Hersh, *Molec. cell. Biochem.* **47**, 35 (1982).
11. S. Wilk, *Life Sci.* **33**, 2149 (1983).
12. A. J. Kenny and S. Maroux, *Physiol. Rev.* **62**, 91 (1982).
13. B. O'Connor and G. O'Cuinn, *Eur. J. Biochem.* **144**, 271 (1984).
14. F. A. Carone, D. R. Peterson, S. Oparil and T. N. Pullman, in *Functional Ultrastructure of the Kidney* (Eds. A. B. Maunsbach, T. Olsen and E. Christensen), p. 327. Pergamon Press, New York (1980).
15. M. Das and R. L. Soffer, *Biochemistry* **15**, 5088 (1976).
16. M. Benuck and N. Marks, *Biochem. biophys. Res. Commun.* **88**, 215 (1979).
17. R. Matsas, I. S. Fulcher, A. J. Kenny and A. J. Turner, *Proc. natn. Acad. Sci. U.S.A.* **80**, 3111 (1983).
18. J. M. Relton, N. S. Gee, R. Matsas, A. J. Turner and A. J. Kenny, *Biochem. J.* **215**, 519 (1983).
19. J. Almenoff and M. Orlowski, *J. Neurochem.* **42**, 151 (1984).
20. W. A. Engelhardt, *Proc. Int. Symp. Enzyme Chemistry (Tokyo)*, IUB Symposium Series, Vol. 2, p. 163. Science Council of Japan, Maruzen, Tokyo (1958).
21. E. M. Danielsen, G. M. Cowell, O. Norén and H. Sjöström, *Biochem. J.* **221**, 1 (1984).
22. A. Helenius and K. Simons, *Proc. natn. Acad. Sci. U.S.A.* **74**, 529 (1977).
23. O. J. Bjerrum, *Analyt. Biochem.* **90**, 331 (1978).
24. B. Malfroy, J. P. Swerts, A. Guyon, B. P. Roques and J.-C. Schwartz, *Nature, Lond.* **276**, 523 (1978).
25. M. A. Kerr and A. J. Kenny, *Biochem. J.* **137**, 477 (1974).
26. I. S. Fulcher and A. J. Kenny, *Biochem. J.* **211**, 743 (1983).
27. I. S. Fulcher, R. Matsas, A. J. Turner and A. J. Kenny, *Biochem. J.* **203**, 519 (1982).
28. A. J. Kenny and I. S. Fulcher, *Ciba Fdn Symp.* **95**, 12 (1983).
29. B. Malfroy and J.-C. Schwartz, *Life Sci.* **31**, 1745 (1982).
30. R. Matsas, A. J. Kenny and A. J. Turner, *Biochem. J.* **223**, 433 (1984).
31. R. Matsas, A. J. Turner and A. J. Kenny, *Fedn Eur. Biochem. Soc. Lett.* **175**, 124 (1984).
32. M. Deschodt-Lanckmann and A. D. Strosberg, *Fedn Eur. Biochem. Soc. Lett.* **152**, 109 (1983).
33. B. P. Roques, M. C. Fournié-Zaluski, E. Soroca, J. M. Lecomte, B. Malfroy, C. Llorens and J-C. Schwartz, *Nature, Lond.* **288**, 286 (1980).
34. B. Malfroy, J. P. Swerts, C. Lorens and J-C. Schwartz, *Neurosci. Lett.* **11**, 329 (1979).
35. S. de la Baume, G. Patey and J-C. Schwartz, *Neuroscience* **6**, 315 (1981).
36. H. Lentzen and J. Palenker, *Fedn Eur. Biochem. Soc. Lett.* **153**, 93 (1983).
37. R. L. Soffer, *A. Rev. Biochem.* **45**, 73 (1976).
38. D. W. Cushman, H. S. Cheung, E. F. Sabo and M. A. Ondetti, *Prog. cardiovasc. Dis.* **21**, 176 (1978).
39. M. Benuck and N. Marks, *J. Neurochem.* **30**, 729 (1978).
40. E. G. Erdös, A. R. Johnson and N. T. Boyden, *Biochem. Pharmac.* **27**, 843 (1978).
41. W. Demmer and K. Brand, *Archs Biochem. Biophys.* **227**, 310 (1983).
42. H. Yokosawa, S. Endo, Y. Ogura and S-I. Ishii, *Biochem. biophys. Res. Commun.* **116**, 735 (1983).
43. M. A. Cascieri, H. G. Bull, R. A. Mumford, A. A. Patchett, N. A. Thornberry and T. Liang, *Molec. Pharmac.* **25**, 287 (1984).
44. F. Chécler, J-P. Vincent and P. Kitabgi, *J. Neurochem.* **41**, 375 (1983).
45. R. B. Harris and I. B. Wilson, *J. biol. Chem.* **258**, 1357 (1983).
46. P. Bûnning, B. Holmquist and J. F. Riordan, *Biochem. biophys. Res. Commun.* **83**, 1442 (1978).
47. P. Bûnning, B. Holmquist and J. F. Riordan, *Biochemistry* **22**, 103 (1983).
48. M. A. Ondetti, B. Rubin and D. W. Cushman, *Science* **196**, 441 (1977).
49. E. Rix, D. Ganten, B. Schull, Th. Unger and R. Taugner, *Neurosci. Lett.* **22**, 125 (1981).
50. M. S. Brownfield, I. A. Reid, D. Ganten and W. F. Ganong, *Neuroscience* **7**, 1759 (1982).
51. A. Arregui, P. C. Emson and E. G. Spokes, *Eur. J. Pharmac.* **54**, 121 (1978).
52. S. M. Strittmatter, M. M. S. Lo, J. A. Javitch and S. H. Snyder, *Proc. natn. Acad. Sci. U.S.A.* **81**, 1599 (1984).
53. J. K. McDonald and A. J. Barrett, *Mammalian Proteases: A Glossary and Bibliography*, Vol. 2. Academic Press, London, in press.
54. K. Hiwada, M. Yokoyama and T. Kokubu, *Biomed. Res.* **2**, 517 (1981).
55. E. D. Wachsmuth, I. Fritze and G. Pfleiderer, *Biochemistry* **5**, 175 (1966).
56. J. M. Hambrook, B. A. Morgan, M. J. Rance and C. F. C. Smith, *Nature, Lond.* **262**, 782 (1976).
57. J. L. Meek, H. Y. T. Yang and E. Costa, *Neuropharmacology* **16**, 151 (1977).
58. A. C. Lane, M. J. Rance and D. S. Walter, *Nature, Lond.* **269**, 75 (1977).
59. C. Gorenstein and S. H. Snyder, *Life Sci.* **25**, 2065 (1979).
60. M. Knight and W. A. Klee, *J. biol. Chem.* **253**, 3843 (1978).
61. R. L. Hudgin, S. E. Charleson, M. Zimmerman, R. Mumford and P. L. Wood, *Life Sci.* **29**, 2593 (1981).
62. S. de la Baume, C. Gros, C-C. Yi, P. Chaillet, H. Marcais-Collado, J. Costentin and J.-C. Schwartz, *Life Sci.* **31**, 1753 (1982).
63. L. E. Geary, K. S. Wiley, W. L. Scott and M. L. Cohen, *J. Pharmac. exp. Ther.* **221**, 104 (1982).
64. M. Hayashi and K. Oshima, *J. Biochem. Tokyo* **84**, 1363 (1977).
65. H. P. Schnebli, M. A. Phillipps and R. K. Barclay, *Biochim. biophys. Acta* **569**, 89 (1979).
66. L. J. Traficante, J. Rotrosen, J. Siekierski, H. Tracer and S. Gershon, *Life Sci.* **26**, 1697 (1980).
67. L. B. Hersh and J. F. McKelvy, *J. Neurochem.* **36**, 171 (1981).
68. L. B. Hersh, *Biochemistry* **20**, 2345 (1981).
69. K-S. Hui, Y-J. Wang and A. Lajtha, *Biochemistry* **22**, 1062 (1983).
70. G. M. Gray and N. A. Santiago, *J. biol. Chem.* **252**, 4922 (1977).
71. A. J. Kenny, A. G. Booth, S. G. George, J. Ingram, D. Kershaw, E. J. Wood and A. R. Young, *Biochem. J.* **157**, 169 (1974).
72. A. Barth, H. Schulz and K. Neubert, *Acta biol. med. germ.* **32**, 157 (1974).
73. T. Kato, T. Nagatsu, K. Fukasawa, M. Harada, I.

Nagatsu and S. Sakakibara, *Biochim. biophys. Acta* **525**, 417 (1978).
74. E. Heymann and R. Rentlein, *Fedn Eur. Biochem. Soc. Lett.* **91**, 360 (1978).
75. C. M. Lee, B. E. B. Sandberg, M. R. Hanley and L. L. Iversen, *Eur. J. Biochem.* **114**, 315 (1981).
76. R. Horsthemke, P. Leblanc, C. Kordon, S. Wattiaux-de Conink, R. Wattiaux and K. Bauer, *Eur. J. Biochem.* **139**, 315 (1984).
77. I. W. Chubb, A. J. Hodgson and G. H. White, *Neuroscience* **5**, 2065 (1980).
78. I. W. Chubb, E. Ranieri, G. H. White and A. J. Hodgson, *Neuroscience* **10**, 1369 (1983).
79. T. J. Miller and I. W. Chubb, *Neuroscience* **12**, 441 (1984).
80. H. S. Cheung and D. W. Cushman, *Biochim. biophys. Acta* **293**, 451 (1973).
81. S. L. Engel, T. R. Schaeffer, B. I. Gold and B. Rubin, *Proc. Soc. exp. Biol. Med.* **140**, 240 (1972).
82. H. Gavras, H. R. Brunner, J. H. Laragh, J. E. Sealey, I. Gavras and R. A. Vukovich, *New Engl. J. Med.* **291**, 817 (1974).
83. D. W. Cushman, J. Pluscec, N. J. Williams, E. R. Weaver, E. F. Sabo, O. Kocy, H. S. Cheung and M. A. Ondetti, *Experientia* **29**, 1032 (1973).
84. H-S. Cheung, F-L. Wang, M. A. Ondetti, E. F. Sabo and D. W. Cushman, *J. biol. Chem.* **255**, 401 (1980).
85. F. Quiocho and W. N. Lipscomb, *Adv. protein Chem.* **25**, 1 (1971).
86. L. D. Byers and R. Wolfenden, *Biochemistry* **12**, 2070 (1973).
87. P. H. Vlasses, R. K. Ferguson and K. Chatterjee, *Pharmacotherapy* **2**, 1 (1982).
88. W. B. Abrams, R. O. Davies and R. K. Ferguson, *Fedn Proc.* **43**, 1314 (1984).
89. H. R. Kaplan, D. M. Cohen, A. D. Essenburg, T. C. Major, T. E. Mertz and M. J. Ryan, *Fedn Proc.* **43**, 1326 (1984).
90. P. S. Wolf, W. S. Mann, J. T. Suh, B. Loev and R. D. Smith, *Fedn Proc.* **43**, 1322 (1984).
91. A. A. Patchett, E. Harris, E. W. Tristram, M. J. Wyvratt, M. T. Wu, D. Taub, E. R. Peterson, T. J. Ikeler, J. ten Broeke, L. G. Payne, D. L. Ondeyka, E. D. Thorsett, W. J. Greenlee, N. S. Lohr, R. D. Hoffsommer, H. Joshua, W. V. Ruyle, J. W. Rothrock, S. D. Aster, A. L. Maycock, F. M. Robinson, R. Hirschmann, C. S. Sweet, E. H. Ulm, D. M. Gross, T. C. Vassil and C. A. Stone, *Nature, Lond.* **288**, 280 (1980).
92. C. Llorens, G. Gacel, J-P. Swerts, R. Perdrisot, M-C. Fournié-Zaluski, J-C. Schwartz and B. P. Roques, *Biochem. biophys. Res. Commun.* **96**, 1710 (1980).
93. R. A. Mumford, M. Zimmerman, J. ten Broeke, D. Taub, H. Joshua, J. W. Rothrock, J. M. Hirsfield, J. P. Springer and A. A. Patchett, *Biochem. biophys. Res. Commun.* **109**, 1303 (1982).
94. J. Almenoff and M. Orlowski, *Biochemistry* **22**, 590 (1983).
95. M-C. Fournié-Zaluski, E. Lucas, G. Waksman and B. P. Roques, *Eur. J. Biochem.* **139**, 267 (1984).
96. A. J. Kenny, in *Proteinases in Mammalian Cells and Tissues* (Ed. A. J. Barrett), p. 393. North-Holland, Amsterdam (1977).
97. R. K. Barclay and M. A. Phillips, *Biochem. biophys. Res. Commun.* **96**, 1732 (1980).
98. H. Umezawa and T. Aoyagi, in *Proteinases in Mammalian Cells and Tissues* (Ed. A. J. Barrett), p. 637. North-Holland, Amsterdam (1977).
99. T. Aoyagi, H. Tobe, F. Kojima, M. Hamada, T. Takeuchi and H. Umezawa, *J. Antiobiot., Tokyo* **31**, 636 (1978).
100. B. P. Roques, E. Lucas-Soroca, P. Chaillet, J. Costentin and M. C. Fournié-Zaluski, *Proc. natn. Acad. Sci. U.S.A.* **80**, 3178 (1983).
101. Z. Vogel and M. Altstein, *Fedn Eur. Biochem. Soc. Lett.* **98**, 44 (1979).
102. S. H. Wilkes, J. O. Baker and J. M. Prescott, *Fedn Proc.* **43**, 1961 (1984).
103. D. K. Meyer and T. Feuerstein, *Trends pharmac. Sci.* **5**, 220 (1984).
104. R. Bouboutou, G. Waksman, J. Devin, M-C. Fournié-Zaluski and B. P. Roques, *Life Sci.* **35**, 1023 (1984).