THE INACTIVATION OF ENKEPHALINS BY BRAIN TISSUE

By

Kevin Pritchard

A thesis submitted for the degree of

Doctor of Philosophy

in the

University of London

Biochemistry Department,

Imperial College.

November 1984

ABSTRACT.

The opioid pentapeptides, leucine and methionine enkephalin, are readily degraded by a variety of peptidases in brain tissues. The breakdown of leucine enkephalin (LE) was investigated *in vitro* by incubating 8nM ^{3}H-(Tyr^1)-LE for 15 min with a preparation of striatal membranes or with viable slices of rat striatum, followed by chromatographic separation and quantification of all four possible labelled products. After incubation of ^{3}H-(Tyr^1)-LE with striatal membranes, the major ^{3}H product was ^{3}H Tyr(54% of total labelled products); the other ^{3}H products were Tyr-Gly-Gly (23%), Tyr-Gly-Gly-Phe (15%) and Tyr-Gly (8%). The dose-response relationships of puromycin and bestatin were investigated over the range 100nM to 1mM, while thiorphan and the novel enzyme inhibitor SQ24994 were tested over the range 100pM to 10µM, in combination with a constant concentration of bestatin of 100µM. 100nM puromycin significantly inhibited aminopeptidase activity by 39%, but the maximum degree of inhibition, achieved at doses of 100µM or 1mM, did not exceed 58%. Bestatin at 1mM completely abolished ^{3}H Tyr formation. Carboxypeptidase activity was significantly inhibited by bestatin (56% inhibition by 100µM; 73% by 1mM) or by 10µM thiorphan alone (59%) or 10µM SQ24994 alone (61%), yet was unaffected by puromycin. The Tyr-Gly-Gly forming activity was significantly inhibited (by 88%) by 10µM SQ24994 in the presence of 100µM bestatin; in the slice preparation also, Tyr-Gly-Gly formation was reduced, by 92%, by this combination. In the membrane preparation, 100µM bestatin with either SQ24994 or thiorphan at concentrations from 100pM to 10µM provided significant inhibition of LE breakdown; the additional protection afforded LE by the SQ24994 or thiorphan resulted from their inhibition of Tyr-Gly-Gly and Tyr-Gly-Gly-Phe

formation. This property of SQ24994 and thiorphan was apparent also in the slice preparation, where 10uM SQ24994 significantly inhibited by 71% the formation of Tyr-Gly-Gly; the combination of 100uM bestatin and 10uM SQ24994 inhibited Tyr-Gly-Gly formation by 92% and Tyr formation by 93%, while LE breakdown was reduced by 88%. The Tyr-Gly forming activity was resistant to all inhibitors tested.

Application of 6·7uM SQ24994 to the <u>in vitro</u> mouse vas deferens bioassay preparation potentiated the effects of concentrations of LE from 1nM to 40nM, suggesting that an SQ24994-sensitive peptidase may reduce the concentration at an opioid receptor of LE at a concentration well below the reported Km values of enkephalin-degrading peptidases.

SQ24994 (64nmol) was injected intra-cerebro-ventricularly into rats <u>in vivo</u>; the brains of treated animals contained 0·56pmol/m ME and 0·47pmol/mg LE, compared to control levels of 0·42pmol/mg ME and 0·30 pmol/mg LE (p 0·05), implicating an SQ24994- sensiti enzyme in the breakdown of enkephalins <u>in vivo</u>. However, neither 10uM SQ24994 nor 100uM bestatin, nor both together, affected the ME or LE content of striatal slices <u>in vitro</u>.

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ABBREVIATIONS AND CHEMICAL FORMULAE

Abbreviations

ACE	angiotensin converting enzyme
AHPA	3-amino-2-hydroxy 4 phenyl acetic acid
BSA	bovine serum albumin
CPA	carboxypeptidase A
CPB	carboxypeptidase B
cpm	counts per minute (of radioactivity)
DAP	dipeptidyl aminopeptidase
dpm	disintegrations per minute (of radioactivity)
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunoadsorbent assay
ESR	external standards ratio (scintillation counting)
GABA	gamma amino butyric acid
HPLC	high performance liquid chromatography
icv	intra-cerebro-ventricularly (injected)
ip	intra-peritoneally (injected)
LDH	lactate dehydrogenase
LE	leucine enkephalin
ME	methionine enkephaline
3MP	3 mercaptopropanyl (derivative of an amino acid)
2NA	2-naphthylamide (derivative of an amino acid)
NADH	nicotinamide adenine dinucleotide (reduced form)
SD	standard deviation
SEM	standard error of the mean
TLC	thin layer chromatography

Trivial chemical names

Amastatin	3-amino 2-hydroxy 4-phenyl butanoyl L-leucyl-L-aspartate
Bestatin	3-amino 2-hydroxy 4-phenyl butanoyl L-leucine
Captopril	2-D-methyl 3-mercapto propanoyl L-proline (SQ14225)
Puromycin	(5)-3'- 2-amino-3-(4-methoxyphenyl)-1-oxopropyl amino -3'-deoxy-N,N-dimethyladenosine
SQ 24994	R,S 3-mercapto 2-methyl propionyl dihydroxyphenylalanine
Thiorphan	D,L-3-mercapto 2 benzyl propionyl glycine
Phosphoramidon	N-(a-L-rhamnopyranosyloxyhydroxy phosphinyl)-L-leucyl-L-tryptophan.

ACKNOWLEDGEMENTS.

I would like to thank Prof. J. Hughes for his invaluable guidance of my PhD. project, and all my colleagues in the team at Imperial College for their support, advice and practical assistance.
A major section of the work described below, concerning the effects of enzyme inhibitors on the enkephalin content of striatal tissues, was carried out in close collaboration with Dr. G. Zamboni, whose unique expertise in the assay technique was crucial to the success of the project, and to whom I am also particularly grateful for much stimulating discussion of experimental problems.
I have been privileged in having access to the unpublished results of Drs.P.M.Caesar and I.Berzetei; their contribution has been acknowledged at the appropriate points in the text.
I am most grateful to Jill Collins, and to Ted Eames, for expert typing of the manuscript.

I N T R O D U C T I O N

1.1 INTRODUCTION

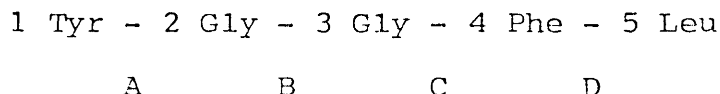
A distinguishing property of a nerve cell or neurone is the ability to carry an action potential. This action potential, propagated along an axon by local changes in the permeability of ion channels in its plasma membrane, must be transmitted across the space (a synapse) between one neurone and the next. Although examples do exist of the direct electrical coupling of neurones, this type of transmission is of little significance in the mammalian central nervous system. Neurones characteristically communicate using chemical messengers or "neuro-transmitters" which are released from the terminal or other specialized region of one neurone, and which diffuse to another neurone. The neurotransmitter molecules then bind to specific receptors on the neuronal surface; the occupation of the receptors then affects the excitability of the target cell, making it more or less likely to initiate an action potential. The nervous system contains a very large number of different substances which affect the excitability of neurones, and these different agents also have different specific receptors, although there is often not a simple one-to-one relationship between transmitter and receptor; the complexity of the system thus enables selective communication between two neurones, even though they may be surrounded by others which may also be signalling, but with different transmitters. The effects of the neurotransmitter are also terminated by a relatively specific mechanism - such as re-uptake (of catecholamines) or enzymic inactivation (of acetylcholine and very probably also of peptides). In the case of the "classical" transmitters, these inactivation processes are very rapid so that a sustained signal requires the sustained release of transmitter, enabling fast switching off of the signal. When, experimentally, drugs are used to inhibit a transmitter-inactivating

system, as when eserine is used to inhibit cholinesterase, the effects of the released transmitter are generally prolonged, and may be more intense. When the normal inactivation system is rendered ineffective, the action of the released substance may be limited only by its diffusion away from its receptors, or by secondary, slower inactivation processes which may not be significant under normal physiological conditions.

Classically, experimental demonstration of a rapid, specific means for the termination of its action has been regarded as evidence that a substance might indeed be a neurotransmitter (Werman, 1966). Recent concepts of neuronal communication envisage diverse types of chemical information-transfer between cells, as demonstrated by the vogue for the term "neuro-modulator". Knowledge of the probable rate of inactivation of a neuroactive agent, coupled with information about the rate of its release in a particular region, may provide clues about whether a substance might be involved in rapid signalling or whether it would be more likely to be involved in regulating the activities of neurones over a longer time-scale. Similarly, studies of the spatial distribution of the sites of release of a neuroactive agent, and of its receptors, are more illuminating when supplemented by data on the distribution of its inactivation system - the two lines of evidence can together suggest that a substance exerts its effects only at discrete synapses, or that the substance remains active sufficiently long to allow its diffusion over a population of cells. Thus, the properties of the inactivating system - its distribution, affinity and specifity - are of vital importance in determining the response to the release of a neuro-active substance.

The work described below concerns the inactivation of the enkephalins. These pentapeptides were the first endogenous opioids to be discovered (Hughes <u>et al</u> 1975) and have since been implicated in the neuronal regulation of a wide range of physiological processes. It has been argued that enkephalins are neurotransmitters at certain synapses (North 1979); they can hyperpolarize populations of neurones in both the peripheral and central nervous systems, inhibiting neuronal firing and transmitter release (Duggan and North, 1983). There is no good evidence for the re-uptake into neurones either of enkephalins or of other intact neuropeptides. These is a consensus that enkephalins are inactivated by enzymatic hydrolysis (Hambrook <u>et al</u>, 1976; Marks <u>et al</u>, 1977; Dupont <u>et al</u>, 1977; Meek <u>et al</u>, 1977; Malfroy <u>et al</u>, 1978; Guyon <u>et al</u>, 1979).

Leucine enkephalin (LE) has the following primary structure:



The four possible enzymic cleavages of LE are as follows:

A   Tyr 1 - Gly 2 cleavage by an aminopeptidase.

B   Gly 1 - Gly 3 cleavage by either an endopeptidase or a dipeptidyl aminopeptidase.

C   Gly 3 - Phe 4 cleavage, either by an endopeptidase or by a dipeptidyl carboxypeptidase.

D   Phe 4 - Leu 5 cleavage by a carboxypeptidase.

In methionine enkephalin (ME), Leu 5 is replaced by Met 5, but the possibilities for enzymic hydrolysis are the same. The cleavage D of either ME or LE yields a tetrapeptide, Tyr-Gly-Gly-Phe, which does retain a low affinity for opioid receptors, although it has less than one-tenth the binding affinity of either pentapeptide; this feeble receptor affinity

is not considered biologically significant. The products of enkephalin cleavage at sites A, B or C, and the smaller peptides generated by further enzymic hydrolysis of primary cleavage products are all devoid of opioid activity (Chang and Cuatrecasas, 1979). In effect, any of the types of enzymic cleavage described above is a potential inactivation process, and enzymes belonging to each of the five categories mentioned have been considered to be involved in the physiological inactivation of enkephalins (Hughes, 1975; Vogel and Altstein, 1977; Swerts et al, 1979 a, b; Benuck and Marks, 1979; Gorenstein and Snyder, 1979; Schwartz, 1983; de la Baume et al, 1983). Enkephalin hydrolyzing enzymes have been the subject of intensive research. The development of this field is described below.

1.2 INACTIVATION OF ENKEPHALINS

Very soon after enkephalins were discovered it became apparent that they were rapidly degraded in the presence of animal body fluids or tissues; this was also inferred indirectly, from the feebleness of their antinociceptive effects even when large doses were injected into the cerebral ventricles (Beluzzi et al, 1976). When radioactively-labelled enkephalins were incubated with rat brain homogenate, hydrolysis was extremely rapid, proceeding by a Tyr - Gly cleavage (Hambrook et al, 1976; Marks et al, 1977). A particle-free supernatant fraction from rat brain possessed similar enzymic activity (Dupont et al, 1977) as did an "opiate receptor" fraction (Meek et al, 1977). In vivo experiments initially confirmed these in vitro results. Meek, Yang and Costa (1977) administered labelled LE intracerebroventricularly to mice and observed that more than 90% had been degraded after 1 min; the half-life of ME in the blood circulation of the rat was found to be 2-4 sec. (Dupont et al, 1977), the major product being tyrosine.

With hindsight it is easy to criticise the use of crude brain homogenates, since the inactivation of enkephalins at or near synapses would be likely to be carried out only by membrane-bound enzymes. The cytoplasm of brain tissue certainly contains a very high level of soluble aminopeptidase activity, many times greater than that bound to membranes (Hersh, 1981) and this soluble enzyme activity masks other types of enkephalin cleavage in a homogenate. In fact Dupont et al (1977) did detect degradation products other than Tyr, while Craves et al (1978) demonstrated that the major metabolite was Tyr - Gly - Gly or Tyr - Gly (or both), when ME was perfused through the rat cerebral ventricular system in vivo. In 1978, Malfroy et al reported the formation of Tyr - Gly - Gly by a washed brain membrane preparation. The enzyme activity responsible was

designated "enkephalinase". That the Tyr-Gly-Gly originated by a primary Gly-Phe cleavage, and not by the sequential activity of a carboxypeptidase, was demonstrated when Guyon et al (1979) identified the primary cleavage product PheMet. An enzyme was already well-known which could cleave enkephalins in this fashion; this was the "angiotensin converting enzyme". Although it at first appeared that enkephalinase and angiotensin converting enzyme might be identical (Swerts et al, 1979; Benuck and Marks, 1979), studies using selective inhibitors (Sullivan et al 1980, Schwartz et al, 1980; Roques et al, 1980) and of regional distribution in the brain (Malfroy et al, 1979; Gorenstein and Snyder, 1980) and of ontogenic development (Schwartz et al, 1980) culminating in the chromatographic separation of the two enzymes (Gorenstein and Snyder 1979, 1980; Benuck and Marks, 1980) have demonstrated that they are in fact distinct.

Patey et al (1981) originally considered that enkephalinase was the sole enzyme responsible for the hydrolysis of synaptically released enkephalins, but it now appears that both enkephalinase and aminopeptidase(s) are important, at least in the in vitro brain slice model (de la Baume et al, 1982, 1983).

Other enzymes do, however, exist which are capable of inactivating enkephalins. Gorenstein and Snyder (1979) described as "enkephalinase B" an enzyme releasing Tyr-Gly from enkephalins; such activity was also detected by de la Baume et al (1983) but was a very minor pathway of inactivation. Other dipeptidyl aminopeptidases have also been found in the brain (Kato et al, 1980).

Finally, carboxypeptidases can degrade enkephalins in vitro (Hughes, 1975) but have not been considered important in vivo; Guyon et al (1979) detected only an

insignificant amount of Tyr-Gly-Gly-Phe formation by striatal membranes.

Subsequent sections describe in more detail aspects of the distribution, specificity and enzymology of enkephalinase, dipeptidyl aminopeptidases, aminopeptidases and carboxypeptidases.

1.3 AMINOPEPTIDASES

1.3.1 DISTRIBUTION OF AMINOPEPTIDASES.

(a) General.

Aminopeptidases capable of hydrolysing enkephalins are widely distributed among body tissues (Erdos et al 1978; Dupont et al, 1977; Ellis and Perry, 1966; Behal et al, 1966; Cushman and Ondetti, 1981). Blood plasma contains aminopeptidase (Dupont et al, 1977; Lane et al, 1977) and the human enzyme has been highly purified by Coletti-Previero et al (1981); in spite of its high Km value for LE of 400μM the enzyme was considered to be capable of hydrolysing enkephalins at a sufficient rate to account for their short half-life in blood. Aminopeptidase activity is also present in cerebrospinal fluid (Lane et al, 1977), although Craves et al (1978) found that when ME was perfused through the cerebral ventricles in vivo, its degradation occurred mainly within brain tissue.

(b) Within brain tissue.

Shaw and Cook (1978) studied the distribution of arylamidase activity among the tissue constituents of brain by means of a light-microscopic histochemical technique and reported finding 6 different aminopeptidases, some of which were differentially associated with particular cell types. Thus, when alanyl 2-naphthylamide was used as substrate, staining was confined to blood vessels, but when leucyl 2-Naphthylamide was used, neuronal cell bodies were also intensely stained. The association of aminopeptidase activity with non-neuronal tissue components means that sub-cellular fractionation experiments and studies of gross regional distribution are difficult to interpret.

(c) Regional distribution in the brain.

Meek et al (1977), Sullivan et al (1978) and also Gorenstein and Snyder (1980) found that the amount of enkephalin degrading aminopeptidase in various regions of rat brain was fairly uniform - in contrast to the marked regional variations in both opioid

receptor and enkephalin concentrations. This uniformity was also apparent in the mouse brain (Malfroy et al, 1979).

(d) Sub-cellular localisation.

Among sub-cellular fractions of brain tissue, the soluble aminopeptidase activity associated with the supernatant (S3) fraction is many times greater than that bound to particulate fractions (Lane et al 1977; Hersh, 1981); much of the activity associated with the synaptosomal fraction was in the occluded cytoplasm, but a significant level of activity was bound to synaptosomal membranes following osmotic lysis (Lane et al, 1977). Membrane bound aminopeptidases exist at the surfaces of intact neuroblastoma cells (Hazum et al, 1979) while washed brain slices possess a high level of aminopeptidase activity (de la Baume et al 1982, 1983). The presence of aminopeptidase on extensively washed preparations of brain membranes has been demonstrated many times (Craves et al, 1978; Malfroy et al, 1979). The results of Lane et al (1977) suggest that the enzyme species found on membranes are different from those in the soluble fraction. It remains to be demonstrated whether one or more of these aminopeptidases is located close to enkephalinergic synapses or opioid receptors; the close functional coupling described by Knight and Klee (1978) has not been investigated further.

1.3.2. PURIFICATION OF ENKEPHALIN-HYDROLYSING AMINOPEPTIDASES.

Several mammalian aminopeptidases have been purified and characterized, including leucine and alanine aminopeptidases, aminopeptidase M, and bovine lens aminopeptidase; Ellis and Perry (1966) studied the arylamidases of bovine pituitary. The possibility that aminopeptidases might be important in the inactivation of enkephalins stimulated new efforts to purify these enzymes from brain tissues; some of the "enkephalin degrading" aminopeptidases isolated in recent years are probably identical to enzymes already purified using other substrates and from other tissues.

Soluble aminopeptidases hydrolysing enkephalins have now been purified from the brains of various

species of mammal: by Traficante et al (1980) from human corpus striatum; from bovine brain by Hersh et al (1981); from rat brain (Schnebli et al, 1979 and Wagner et al, 1981) and monkey brain (Hayashi et al, 1977). The aminopeptidases from different animal species appear to be different enzymes since they have Km's for enkephalin ranging from 3 µM to 170 µM, and exhibit different specificities for 2-naphthylamide model substrates. It is more likely that in each case the purified enzyme is but one of several aminopeptidases present in a single animal species, than that their diversity is attributable only to inter-specific differences.

Fewer membrane-bound aminopeptidases have been purified, although aminopeptidase M occurs in a membrane-bound form (George and Kenny, 1973). Hersh (1981) purified two distinct aminopeptidases from rat brain membranes. One enzyme had a much lower Km for enkephalins than the other; only the high affinity type was readily inhibited by puromycin, and this type was responsible for almost all the enkephalin degrading activity of the membrane preparation.

1.3.3. AMINOPEPTIDASES: ENZYMOLOGY AND INHIBITOR STUDIES

The molecular weights of purified aminopeptidases (section 1.3.2.) are around 100 Kd, except for the human enzyme, which has Mw 68 Kd. Reported pH optima are around pH 7.

Aminopeptidases are exopeptidases characterized by an ability to cleave single amino acids from the N termini of peptides. For reasons of experimental convenience, amino acid 2-naphthylamides are very widely used as model substrates; in such cases it is more precise to refer to "arylamidase" activity - this is not necessarily equivalent to aminopeptidase activity (Behal et al, 1966; Marks et al, 1968). The sizes of peptides from which amino acids can be cleaved varies from enzyme to enzyme. Some aminopeptidases can also act as di - and tri - peptidases (Hersh et al, 1981). Those aminopeptidases purified and analyzed in detail have all contained catalytically active zinc (Cushman and Ondetti,

1981). Wagner et al (1981) demonstrated spectrophotometrically that their soluble rat brain aminopeptidase contained 1 mole of zinc per mole of protein. It seems likely that the other "enkephalin degrading" aminopeptidases are also zinc metallopeptidases (Hersh, 1982), although evidence from inhibition studies using group-specific reagents has been ambiguous. Metal-chelating agents such as EDTA or 1,10 phenanthroline have been found to be inhibitory on all the aminopeptidase activities on which they have been tested (Marks et al, 1968; Lane et al, 1977; Traficante et al 1980; Sullivan et al, 1980; Hersh and McKelvy, 1981), while the hydroxamate class of aminopeptidase inhibitors (Hudgin et al, 1981; Coletti-Previero et al, 1982) presumably chelate zinc at the active site, as has been demonstrated in the case of thermolysin by Nishino and Powers (1979) and Holmquist and Vallee (1979). However several investigators have reported the inhibition of preparations of aminopeptidase by reagents specific for thiol groups, such as parachloromercuriphenylsulphonic acid (Ellis and Perry, 1966; Meek et al 1977; Malfroy et al, 1978) while the enzymes studied by Traficante et al (1980) and by Hersh and McKelvy (1981) were sensitive to parachloromercuribenzoic acid as well as to metal-chelating agents. Available evidence suggests that these aminopeptidases are metallopeptidases but that they contain thiol groups essential for activity, rather than that they are thiol peptidases.

Three compounds have been widely used empirically to suppress the breakdown of enkephalins by aminopeptidases. These are bacitracin, puromycin and bestatin. Bacitracin has been used to protect enkephalins released from brain slices (Iversen et al, 1978) and increases the apparent affinity of enkephalins in receptor-binding assays (Miller et al, 1977). Bacitracin also inhibits "enkephalinase" activity (Sullivan et al, 1980) but is of low potency with an IC 50 of 10-20 μm against either type of enzyme (Hudgin et al, 1981). It has been known for

several years that puromycin could inhibit certain aminopeptidases (Ellis and Perry, 1966). Puromycin at 100 μM completely inhibited the hydrolysis of LE by a crude homogenate of rat brain, its IC 50 being 200nM (Vogel and Altstein, 1978). Schwartz et al (1980) showed that the compound inhibited the degradation of LE by brain membranes; also in brain membranes Hudgin et al (1981) reported an IC50 of 120μM against the formation of Tyr from LE, and an IC50 of 20mM against enzymes forming Tyr-Gly-Gly. However, puromycin does not inhibit all aminopeptidases. Commercial leucine aminopeptidase was resistant (Vogel and Altstein, 1978) as was the enzyme purified from human plasma by Coletti-Previero et al (1981). Hersh (1981) reported that one of two purified rat brain aminopeptidases was resistant to puromycin inhibition. It is particularly interesting that puromycin appears to be incapable of protecting from degradation enkephalins released from brain slices in vitro (Patey et al, 1981) - this is inconsistent with the statement of Hersh (1981) that it was the puromycin-sensitive species of enzyme which was more active in enkephalin breakdown. Bestatin is a derivative of the amino acid 3 - amino - 2 - hydroxy 4 - phenyl butanoic acid (AHPA), viz., AHPA - L - Leu, and is a potent inhibitor of rat brain particulate-bound aminopeptidase (IC 50, 200 nM), as is the related compound amastatin (AHPA - L - Leu - L - Asp), the IC 50 of which is 10 nM (Barclay and Phillips, 1980). Although amastatin may be more potent, bestatin has been investigated more thoroughly. Numerous AHPA containing depeptides have been screened as aminopeptidase inhibitors (Cushman and Ondetti, 1981). The inhibitor-specificity of the soluble rat brain enzyme purified by Wagner and Dixon (1981) paralleled its substrate-specificity for amino-acyl 2-naphthyl-amides (Schnebli et al, 1979). Unlike puromycin, bestatin inhibits both leucine aminopeptidase and aminopeptidase B. De la Baume et al (1982, 1983) have shown that bestatin, unlike puromycin, potently inhibits that aminopeptidase activity which contributes to the

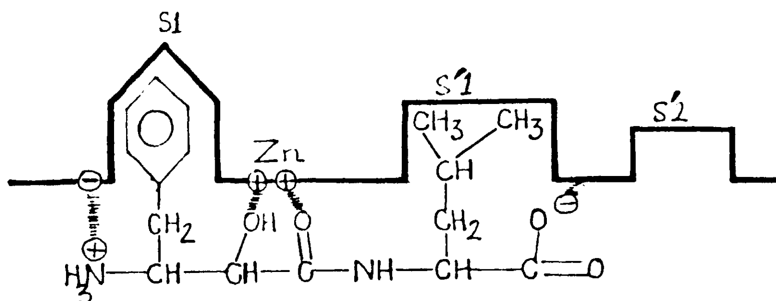


Fig. 1.3.3: **Model for the binding of the inhibitor bestatin to a zinc aminopeptidase.**

This diagram is based on a model proposed by Cushman and Ondetti (1981). The Zn-containing active site of an aminopeptidase has been presumed to be functionally analogous to that of carboxypeptidase A (Quiocho and Lipscomb, 1971) while the different substrate specificity arises by alterations in the sequence of the protease "sub-sites": S1, binding substrate aminoacid side-chains which are N-terminal to the scissile bond; and sub-sites S'1 and S'2 binding substrate amino acid residues C-terminal to the scissile bond. The adjacent hydroxyl and carbonyl groups of bestatin may chelate the zinc ion at the active site in a similar way to the binding of hydroxamate derivatives to the thermolysin active site (Nishino and Powers, 1979).

degradation of enkephalins released from brain slices.

There seems to be no firm evidence that the AHPA series of inhibitors interacts with zinc ion at the active sites of aminopeptidases. However, the hydroxyl and carbonyl functions are borne on adjacent carbon atoms, and may chelate the zinc ion in a similar way to that demonstrated for the hydroxamate group and Cushman and Ondetti (1981) have offered a tentative model of bestatin binding (figure 1.3.3.), similar to the models for the binding of inhibitors to other zinc-metallopeptidases (section 1.5.6)

1.4. DIPEPTIDYL AMINOPEPTIDASES.

Enzymes in this class cleave dipeptides from the unsubstituted amino termini of peptides. Gorenstein and Snyder (1979) described as "enkephalinase B" an enzyme cleaving the N terminal Tyr-Gly from enkephalins. De la Baume et al (1982, 1983) found that Tyr-Gly was only a very minor product of the hydrolysis of enkephalins incubated with brain slices or brain membranes. Hersh et al (1982) stated that by far the larger fraction of Tyr-Gly generating enzyme activity was soluble and cytoplasmic. The properties of "enkephalinase B" reported by Gorenstein and Snyder (1979) seem not to correspond with those of known dipeptidyl aminopeptidases of which at least four types exist (Kato et al, 1980), but its substrate specificity has not been investigated. Although it is unlikely that this class of enzymes are important in enkephalin inactivation, dipeptidyl aminopeptidase II has been cited as a possible "neuropeptidase" by Gorenstein et al (1981).

1.5. ENKEPHALINASE

1.5.1. NOMENCLATURE

"Enkephalinase" is an enzyme, distinct from angiotensin converting enzyme, which hydrolyses enkephalins at the Gly 3 Phe 4 bond. The term has been particularly associated with the investigations of Schwartz and co-workers. Subsequent developments, summarised below, have cast doubt on the substrate specificity and restricted physiological role implied by the name "enkephalinase". However, where research workers have described their enzyme activity as "enkephalinase", that name has been retained in the text.

1.5.2. DISTRIBUTION OF ENKEPHALINASE

(a) General.

Llorens and Schwartz (1981) reported that enkephalinase activity was present in several peripheral tissues, and that the level of activity in some tissues, e.g., kidney, lung, thyroid and salivary gland, was higher than that in the corpus striatum, the brain tissue richest in the enzyme. Enkephalinase was originally described as a "dipeptidyl carboxypeptidase". However, the enzyme activity studied by the Schwartz group does in fact cleave amidated substrates (Roques et al, 1982). This finding raises the possibility that the enzyme might be identical with an endopeptidase described by George and Kenny (1973) and purified and characterised by Kerr and Kenny (1974 a,b) from the brush border membranes of the kidney; this enzyme cleaves peptide bonds to the amino terminal side of hydrophobic residues.

Malfroy and Schwartz (1982 a) isolated from a microsomal fraction of rat kidney an enzyme which they regard as identical to that from brain. The kidney enzyme hydrolysed the B chain of insulin as did that studied by Kerr and Kenny (1974 a) and possessed endopeptidase activity; the inhibition of the enzyme by phosphoramidon and by thiorphan was demonstrated.

Orlowski and Wilk (1981) purified a cation-sensitive neutral endopeptidase from bovine pituitary, which bore many similarities to the enzyme of Kerr and Kenny (1974 a, b) although some discrepancies were noted. Almenoff _et al_ (1981) compared the substrate and inhibitor specificities and kinetic properties of this pituitary enzyme with those of the activity associated with a particulate membrane fraction; it was concluded that the enzymes were probably identical. In particular, the enzyme of synaptic membranes hydrolysed the endopeptidase substrate glutaryl - Ala - Ala - Phe - 2NA and the distribution of this endopeptidase activity between brain regions was very similar to that reported by Malfroy _et al_ (1979) and by Gorenstein and Snyder (1980) for enkephalinase activity.

Benuck and Marks (1980) used an immuno-affinity column to separate angiotensin converting enzyme from a distinct "peptidyl dipeptidase B" capable of hydrolysing enkephalins. Several properties of this enzyme resembled those reported for the "classical" brain enkephalinase of the Schwartz group. Benuck, Berg and Marks (1981) detected large amounts of this activity in both lung and kidney.

Fulcher _et al_ (1982) compared the properties of bovine kidney neutral endopeptidase (the activity originally investigated by Kerr and Kenny, 1974 a) with those of synaptic membranes: thiorphan and phosphoramidon are equipotent on kidney endopeptidase, as inhibitors of the hydrolysis of either LE or insulin B chain. In further experiments, Matsas _et al_ (1983) showed that phosphoramidon had similar effects on synaptic membranes and on the purified kidney enzyme. The enzyme from either tissue source inactivated substance P forming a characteristic, complex pattern of peptide fragments; substance P hydrolysis was in both cases inhibited by phosphoramidon. Finally, a specific anti-serum raised against the purified kidney enzyme inhibited hydrolysis of either substance P or LE; the titration curve of antibody

concentration versus activity was the same for both enzymes.

Both Malfroy and Schwartz (1982 a) and Matsas et al (1983) have expressed the opinion that the kidney and brain enzymes are the same; in view of the very impressive similarities, particularly the antigenic similarity, it seems practically certain that the two tissues have an endopeptidase species in common.

Thus, enkephalinase is an endopeptidase of broad specificity and, probably, a wide distribution among body tissues. It is not at present clear whether only one neutral endopeptidase has been studied by groups working in this field. Benuck, Berg and Marks 1982 (a) detected some differences in specificity between the enzyme activity of synaptosomal membranes (Benuck, Berg and Marks, 1982 b) and the metalloendopeptidase which they have highly purified from rat brain and kidney. Only the latter, purified enzyme cleaved ME Arg 6 Phe 7 at the Gly 3 Phe 4 bond. Yang et al (1981) likewise found striatal microsomes incapable of this cleavage of ME Arg 6 Phe 7. Benuck et al consider the purified endopeptidase to be identical to that previously detected in rabbit brain and kidney (see above). The results of Fulcher et al (1982) suggest that striatal membranes might possess an endopeptidase additional to that common to both kidney and striatum; whereas the inhibitors thiorphan and phosphoramidon completely suppress the breakdown of enkephalin by both kidney and striatal preparations, these compounds inhibited the hydrolysis of the B chain of insulin only by the purified kidney enzyme. The metalloendopeptidase purified by Mumford et al (1981) was competitively inhibited by angiotensin I or II, corticotropin, somatostatin, ME, LE or bradykinin but did not recognise oxytocin; similar enzyme activity was detected in numerous other tissues (Mumford et al 1980). Oxytocin was a substrate for the endopeptidase of pituitary and brain investigated

by Almenoff et al (1981). The patterns of cleavage of bradykinin and neurotensin reported by Almenoff et al (1981) indicate an enzyme of different specificity from the neutral endopeptidase investigated by Wilk and Orlowski (1980); the latter enzyme, moreover, hydrolysed angiotensin II in a different manner from the human kidney "enkephalinase" described by Gafford et al (1983). At the moment it is uncertain whether these discrepancies are wholly the consequence of experimental variations, or whether more than one neutral endopeptidase exists; Marks and Benuck (1983) have remarked that the specificity of angiotensin converting enzyme is somewhat altered when the enzyme has been solubilized from its normal, membrane-bound form.

It is probable that a neutral endopeptidase plays an important part in terminating the actions of enkephalins in brain tissues, and perhaps also in some peripheral tissues such as gut or vas deferens. It is unlikely that this can be the sole function of the endopeptidase activity found in a diverse range of peripheral tissues, such as lung, kidney and thyroid.

(b) Within brain tissues.

To date, no microscopic studies of the localization of neutral endopeptidase/enkephalinase have been reported. The existence of a specific antiserum (Matsas et al, 1983) and perhaps the development of fluorogenic substrates and of a photoaffinity label (Roques et al, 1982) may be steps on the way to such an approach. That the enzyme is borne by neurones has been suggested by lesion studies.

A major pathway of enkephalinergic neurones runs from the substantia nigra to the corpus striatum (Cuello, 1983). Microinjection of the specific lesioning agent, 6 - hydroxydopamine, into the lateral hypothalamic area selectively depletes nigrostriatal depaminergic neurones bearing opioid receptors, (Pollard et al, 1977); about 20% of opioid receptor binding activity disappears with an equivalent loss of enkephalinase

activity (Malfroy et al, 1979). Llorens-Cortes et al (1979) demonstrated that opioid receptors are present on nigrostriatal dopaminergic neurones and also on intrastriatal (perhaps GABA-ergic) neurones. That both receptor and enzyme are borne on post-synaptic neurones is suggested by the parallel losses of both opioid receptor binding and enkephalinase activity following injection of kainic acid directly into the corpus striatum (Malfroy et al, 1979). A proportion of the enzyme may be associated with tissue elements other than neurons. Lentzen et al (1983), studying both bulk-prepared cells and tissue cultures, reported that enkephalinase A activity can be detected on astrocytes and other glial cells rather than on neurones.

(c) Subcellular localization in the brain.

De la Baume et al (1981) demonstrated that the distributions of opioid receptor binding and of enkephalinase activity in rat brain were parallel among primary fractions of either cerebral cortex or corpus striatum. The levels of both markers were about two-fold higher in the microsomal P3 fraction than in the primary mitochondrial fraction (P2), consistent with the presence of both enzyme and receptor on both axonal and perikaryal surfaces of neurones, or even on glial cell surfaces. Further sub-fractionation of P2 after osmotic lysis showed that both markers were closely associated with synaptosomal membranes and "ghosts". This distribution of enkephalinase, like that of the opioid receptor, is consistent with an involvement in synaptic transmission.

(d) Regional distribution within brain.

Enkephalinase has a markedly **heterogeneous distribution.** The highest levels, found in the corpus striatum, are three times greater than the levels in the cerebellum (Malfroy et al 1979). Schwartz et al (1980) reported that the levels of enkephalinase activity and of opioid receptors were strikingly correlated in six mouse brain regions. The results of Sullivan et al (1979) and of Gorenstein and Snyder (1980) indicated a similar regional distribution in rat

brain, as did those of van Veldhoven and Carton (1982) who also demonstrated that enkephalinase activity was higher in dorsal than in ventral spinal cord - in parallel with known variations in ME and receptor levels. However, Llorens _et al_ (1982) found that when human brain was finely dissected into more precise regions, the correlation between the enzyme and the receptor was not so close - it was surmised that enkephalinase might rather be distributed in parallel with enkephalins.

1.5.3. MOLECULAR PROPERTIES OF ENKEPHALINASE.

The enzyme is monomeric with a molecular weight in the range 90-95 Kd (Kerr and Kenny 1974 a; Orlowski and Wilk, 1981; Malfroy and Schwartz, 1982 a; Gafford _et al_ 1983). Binding to lectins has frequently been employed as a method of purification (Rush and Hersh 1982; Malfroy and Schwartz 1982 a) and indicate that the molecule bears carbohydrate chains; Gafford _et al_ (1983) estimate that the molecule contains 10% neutral sugars. Kerr and Kenny (1974 b) used a staining technique to demonstrate that the kidney neutral endopeptidase contained carbohydrate. Differences in carbohydrate composition probably account for part of the molecular heterogeneity of "enkephalinases". Gorenstein and Snyder (1980) reported that rat brain enkephalinase (called by them "enkephalinase A" to distinguish it from "enkephalinase B" which is probably a dipeptidyl aminopeptidase) could be resolved into two peaks of activity by anion exchange. This was confirmed by Rush and Hersh (1982); the charge difference between the two forms (A1 and A2) was determined by chromatofocussing which yielded apparent pI's of 5.5 for A1 and 5.2 for A2. Both A1 and A2 could be further fractionated into two components, only one of which bound to wheat germ lectin. Thus, four forms exist; A1, 2 and A2, 2 bind to the lectin. Rush and Hersh consider that A1, 2 and A2, 2 have either a higher sialic acid content, or carbohydrate chains which are more highly branched. No differences were found when the four enzyme forms were screened against enzyme

inhibitors - in contradiction to the report of Gorenstein and Snyder (1980) that the lower pI form (A2) was inhibited by phenylmethyl sulphonylfluoride. The Km values for LE hydrolysis were; A1, 1 - 78 μM; A1, 2 - 79 μM; A2, 1 - 124 μM; A2, 2 - 63 μM. The significance of this heterogeneity is obscure, although it is conceivable that the differences in carbohydrate structure might influence the behaviour of the enzyme in the membrane - the glycan portion of the molecule is important in orienting peptidases in the kidney brush border membrane (Booth and Kenny, 1980). Rush and Hersh (1982) speculate that the multiple forms of enkephalinase might be differentially associated with different types of opioid receptor.

1.5.4. SUBSTRATE SPECIFICITY OF ENKEPHALINASE.

It is now well established that the enzyme is an endopeptidase, and not a dipeptidyl carboxypeptidase as was originally thought (Schwartz et al, 1980); this has been demonstrated not only by the hydrolysis by the enzyme of artificial substrates with blocked C termini, such as Dansyl - DAla - Gly - pNO_2 - Phe - Gly - NH_2 (Roques et al, 1982) but also by its action on naturally occuring peptides, including the B chain of insulin (Malfroy et al, 1982 a, b) and angiotensin II (Gafford et al, 1983) which is cleaved into two tetrapeptides, and several other substrates, a selection of which is presented in figure 1.5.4. In spite of this ability to function as an endopeptidase, amidation of the C terminus of enkephalins markedly reduces their affinity for the enzyme (Fournie-Zaluski et al, 1979); this preference for a free carboxyl terminus has been related to the presence of a free Arg residue in the active site (Malfroy et al, 1982 b). It has been pointed out (Matsas et al, 1983) that numerous neuropeptides have blocked N and/or C termini, precluding exo- or endo- peptidase inactivation.

The specificity of the enzyme is obviously broad; it has been likened to thermolysin (Almenoff et al,

1981) in its ability to cleave a variety of substrates to the N terminal side of aromatic or large hydrophobic amino acid residues. In view of its general distribution among body organs, its wide specificity and the likelihood that its physiological functions are not solely connected with the inactivation of enkephalins, Matsas et al (1983) proposed that the name, "endopeptidase 24.11" would be less perjorative that "enkephalinase". **Figure 1.5.4.** illustrates the mode of hydrolysis of some biological peptides. It is worth noting that although angiotensin I is a potent inhibitor of LE hydrolysis by "enkephalinase" (Fournie-Zaluski et al, 1979), this enzyme is incapable of cleaving the Phe 8-His 9 bond to generate the vasoactive octapeptide angiotensin II (Benuck and Marks 1980; Gafford et al, 1983). The peptidase appears ineffective at cleaving intact proteins such as casein or insulin (Kerr and Kenny 1974 a, b).

The affinity of the enzyme for enkephalin has been controversial, owing to the initial report of Malfroy et al (1978) that the Km was in the nM range; however this group revised their estimate of the Km of LE to 22 µM (Fournie-Zaluski et al, 1979). All other investigators have recorded Km values in the uM range although the various estimates vary by about 2 orders of magnitude (table 1.5.4.).

FIGURE 1.5.4.

SUBSTRATE SPECIFICITY OF ENKEPHALINASE.

Naturally occuring peptide substrates.

1) [MET]^5 ENKEPHALIN Arg 6 Phe 7 (Marks and Benuck, 1983)

Tyr Gly Gly↓Phe Met Arg Phe

2) DYNORPHINS. (Marks and Benuck, 1983)

Gly^3 Phe 4 cleavage occurs in dynophins of all chain lengths up to 17 residues; however, the velocity of hydrolysis decreases with increasing Mw.

3) ANGIOTENSIN II (Gafford et al, 1983)

Asp Arg Val Tyr↓Ile His Pro Phe

4) OXYTOCIN (Almenoff et al, 1981)

Cys Tyr Ile Gln Asn Cys Pro↓Leu Gly - NH_2

5) BRADYKININ (Almenoff et al, 1981)

Arg Pro Pro Gly Phe Ser Pro↓Phe Arg

6) NEUROTENSIN (Almenoff et al, 1981, Checler et al, 1983)

pGlu Leu Tyr Glu Asn Lys Pro Arg Arg Pro↓Tyr↓Ile Leu

(Only one of the two cleavages indicated occurs in one molecule of substrate).

7) SUBSTANCE P (Matsas et al 1983)

Arg Pro Lys Pro Gln Gln↓Phe↓Phe Gly↓Leu Met NH_2

8) CHOLECYSTOKININ-8 (Deschodt-Lanckman et al, 1983)

Asp Tyr Met Gly Trp Met Asp Phe

9) PHYSALAEMIN (Matsas et al, 1984)

Glu Ala Asp Pro Asn Lys Phe Tyr Gly Leu Met-NH_2

1.5.5. THE ACTIVE SITE OF ENKEPHALINASE ; INHIBITOR STUDIES.

Kerr and Kenny (1974 b) determined spectroscopically that their neutral endopeptidase contained 1 mol Zn per mol enzyme. Other workers have used EDTA to inactivate their preparations of the endopeptidase/"enkephalinase" and attempted to restore activity by adding various metal ions. Orlowski and Wilk (1981) found that their pituitary endopeptidase was re-activated most effectively by zinc. Zinc also reactivated a striatal membrane preparation treated by Schwartz et al (1980) with the chelating agent o-phenanthroline. Sullivan et al, (1980) considered cobalt a more effective ion. However, no metalloprotease has so far been shown to contain an ion other than zinc at its active site (Cushman and Ondetti, 1981), although active enzymes can often be prepared with other ions. That a metal ion is important for catalysis has been confirmed by several workers who have observed inhibition of enzyme preparations by metal chelators including EDTA, glutathione, dithiothreitol, o-phenanthroline and dithizone (Almenoff et al, 1981; Orlowski and Wilk, 1981; Benuck and Marks, 1980; Benuck, Berg and Marks, 1981; Gorenstein and Snyder, 1979, 1980; Schwartz et al, 1980; Sullivan et al, 1980). The enzyme appears resistant to classical inhibitors of serine proteases (such as di-isopropylfluorophosphate or phenyl-methylsulphonyl fluoride) or thiolprotease inhibitors including iodoacetate, p-hydroxymercuribenzoate, N-ethyl maleimide and p-chloromercuriphenyl sulphonate (Almenoff et al, 1981; Gorenstein and Snyder, 1979, 1980; Sullivan et al, 1980; Kerr and Kenny 1974 a, b). It seems clear that the enzyme is a metalloprotease, almost certainly containing a catalytic zinc atom.

Enkephalinase has been generally described as a "neutral" protease. Some authors have reported pH optima of 7 to 7.5 (Orlowski and Wilk, 1981; Benuck, Berg and Marks, 1981) but others have reported a value of pH 6.5 (Gorenstein and Snyder, 1979; Rush and Hersh 1982), while Kerr and Kenny, 1974(b)

TABLE 1.5.4.

Km VALUES OF ENKEPHALINS ON THE NEUTRAL ENDOPEPTIDASE "ENKEPHALINASE".

SOURCE			SUBSTRATE ME μM	LE μM
RAT CORPUS STRIATUM MEMBRANES				
Fournie Zaluski *et al*	1979		1.4	22
Almenoff *et al*	1981		40	
PURIFIED RAT BRAIN "ENKEPHALINASE"				
Rush and Hersh	1982	A1, 1		78
		A1, 2		79
		A2, 2		124
		A2, 2		63
Marks and Benuck	1983			50
Benuck and Marks	1980			140
PURIFIED KIDNEY ENDOPEPTIDASE				
Benuck, Berg and Marks	1981	(Rabbit)		80
Gafford *et al*	1983	(Human)		70
Malfroy and Schwartz	1982	(Rat)	1.15	
Schwartz *et al*	1983	(Rat)	8	
PITUITARY ENDOPEPTIDASE				
Almenoff *et al*	1981		40	

The "Km" values above were obtained by inhibition of labelled enkephalin breakdown by unlabelled enkephalin substrate. Matsas *et al* (1984) obtained the following values, observing strict Michaelis-Menten conditions:

SUBSTRATE	Km/μM
ME	86
LE	62
Substance P	32
Bradykinin	92
CCK-8	67

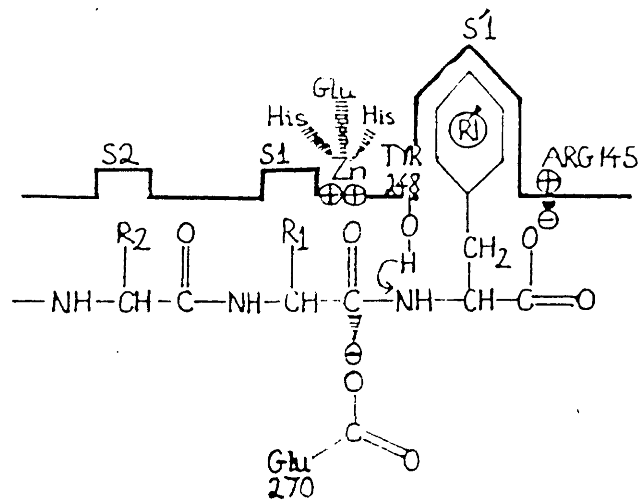
found a pH of 6 to be optimal. The enzyme seems not to have a very narrow pH range. The pH optima are sufficiently high to clearly distinguish enkephalinase from the "acid peptidases" such as cathepsin D, which apparently have a quite different catalytic mechanism.

1.5.6. ENKEPHALINASE AS A ZINC METALLOPEPTIDASE.

The pancreatic digestive enzyme, carboxypeptidase A has been sequenced (Bradshaw et al, 1969; Bradshaw, 1969) and was the first metalloenzyme to be understood, structurally and mechanistically (reviewed by Quiocho and Lipscomb, 1971). Enkephalinase has been assumed to possess an active site homologous to that of carboxypeptidase A (CPA), as has angiotensin converting enzyme (ACE); presumably a family of enzymes has arisen by evolutionary divergence, where the catalytic mechanism has been conserved while mutations have given rise to changes of sequence which can confer different specificities to different enzymes of the same class. This concept has been much discussed in relation to the serine family of protease (Hartley et al, 1965). Just as the bacterial subtilisins are believed to have separate evolutionary origins from the mammalian serine proteases, i.e., to have arisen by convergent evolution (Robertus et al, 1972 a, b) so the Zn protease thermolysin from Bacillus thermoproteolyticus possesses an active site functionally similar to that of CPA (Kester and Matthews, 1977). Enkephalinase has been likened to thermolysin (section 1.5.4.). The binding of phosphoramidon to thermolysin has been elucidated by X-ray crystallography (Weaver, Kester and Matthews, 1977). A series of 3-mercapto propanoyl amino acids has been developed as inhibitors of CPA, ACE and enkephalinase; the binding to thermolysin of the inhibitor 2-benzyl 3-mercapto propanoyl alanyl glycinamide has been analysed crystallographically by Monzingo and Matthews (1982). Analogies between enkephalinase and thermolysin

may be a valuable adjunct to those drawn with CPA, although the details of its structure will not necessarily be identical. The order in the primary sequences of corresponding amino acid residues crucial for binding and catalysis is different in CPA and thermolysin (Kester and Matthews, 1977) and there are significant differences in the active sites (Weaver, Kester and Matthews, 1977). The analogy with CPA provided the rational basis for the design of ACE and enkephalinase inhibitors. The diagram shows the binding of a polypeptide to CPA; four subsites (S1 to S4) bind R groups on the N terminal side of the scissile bond and a subsite S1(Figure 1.5.6.a: the "hydrophobic pocket") confers specificity for C terminal aromatic residues. This nomenclature for the subsites will be retained when discussing the other enzymes. The catalytic mechanism of CPA probably involves the donation of a proton by Tyr 248 (acting as a "general acid") to the N of the scissile peptide bond and an attack by a glutamate residue (acting as a "general base") on the carbonyl carbon atom of the scissile bond - the latter C atom would bear a partial positive charge as a result of the polarization of the carbonyl group by the double positive charge on the zinc ion. A similar mechanism is favoured by Weaver, Kester and Matthews for thermolysin (where the His 231 residue replaces Tyr 248 as the proton donor). The precise mode of attack of the Glu 270 (CPA) or Glu 143 (thermolysin) is controversial (for discussion, see Kester and Matthews, 1977; Quiocho and Lipscomb, 1971) and may be different in the two enzymes (Weaver, Kester and Matthews, 1977).

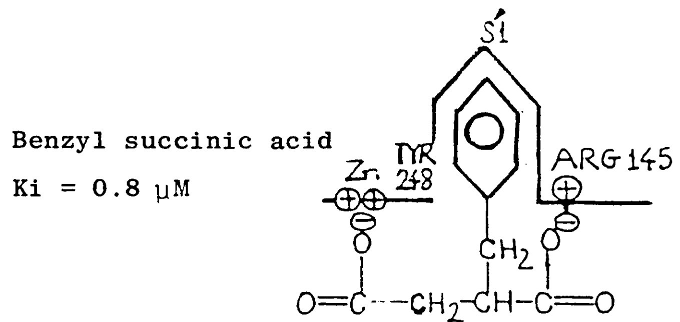
Figure 1.5.6.a represents the binding to CPA of two inhibitors; benzyl succinic acid (Byers and Wolfenden 1973) and a much more potent and specific agent, 2-benzyl 3-mercapto propionic acid (2 benzyl MPA) developed by Ondetti, Condon et al (1979); the substitution of a sulphydryl group for the carboxyl profoundly enhances the inhibitory potency.



Binding of inhibitors to carboxypeptidase A

Benzyl succinic acid

Ki = 0.8 μM



3-Mercapto benzyl

succinic acid

Ki = 11 nM

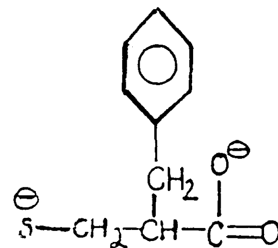


Fig. 1.5.6(a): Model for the binding of a peptide substrate to carboxypeptidase A.

The assumption that ACE has an active site like that of CPA enabled Ondetti, Rubin and Cushman to design a potent ACE inhibitor; the success of this approach can be taken as circumstantial evidence supporting their original assumptions. Two differences were assumed between the ACE and CPA active sites:

(i) ACE is a dipeptidyl carboxypeptidase, so that the distance between the cationic carboxyl binding site and the zinc atom should be greater than that in CPA by approximately the length of one amino acid residue. Since succinic acid derivatives were known to be CPA inhibitors, succinyl amino acids were the basis for development of an ACE inhibitor.

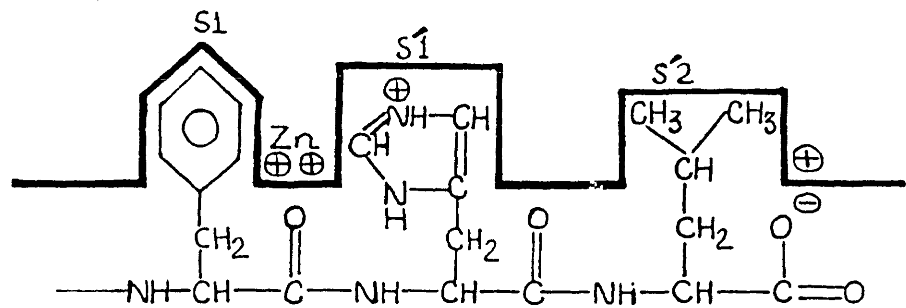
(ii) Carboxypeptidase A subsite S1 is a hydrophobic pocket; angiotensin I has the C terminal sequence:

-Pro Phe His Leu OH

the active octapeptide angiotensin II being generated by a Phe/His cleavage. The potent peptide ACE inhibitors isolated and sequenced from snake venom by Ondetti et al (1971) all have the C terminal sequence Ile Pro Pro as has the clinically effective nonapeptide "Teprotide" (SQ 20881). The phenyl ring of the CPA inhibitor was therefore omitted; succinyl proline derivatives were found to be selective ACE inhibitors. Further modifications led to the synthesis of 2-D-methyl 3-mercapto propanoyl-L-proline, "Captopril" (SQ 14,225) of IC 50 about 20 nM *in vitro* (Ondetti et al, 1977). The model of the ACE active site developed by Ondetti et al (1977) is figure 1.5.6.b.

This background information enabled Roques and co-workers to set about the logical design of a brain "enkephalinase" inhibitor. Initially, a large series of dipeptides was screened (Llorens et al, 1980) - an aromatic N terminal amino acid was crucial for potency. Thus of an X-Ala series, Phe Ala, Tyr Ala and Trp Ala were most potent. The specificity for the C terminal amino acid was less stringent; XPhe, XTrp, XGly and XAla were all

Substrate: Angiotensin I



Inhibitor: Captopril

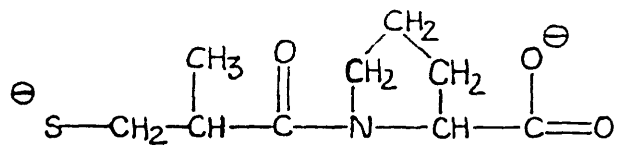


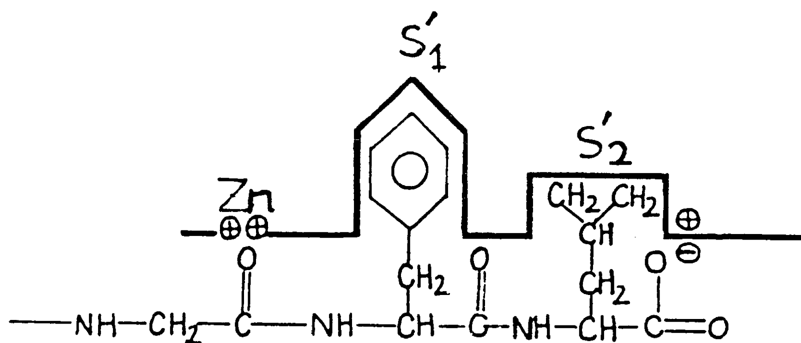
Fig. 1.5.6.(b): Model for the binding of substrate or inhibitor to angiotensin converting enzyme.

potent with XLeu rather less so (X was Phe or Tyr). However, acidic or basic residues, or XPro dipeptides were far less potent; the poor inhibition by Phe Pro demonstrates a difference between the specificities of ACE and enkephalinase (SQ 14225 is a much less potent enkephalinase than ACE inhibitor). The preferences of enkephalinase for the N and C terminal amino acids, respectively, can be described as the specificities of the putative S'1 and S'2 subsites. Finally, the lower inhibitory potencies of C - terminally esterified or amidated enkephalins (Fournie-Zaluski et al, 1979) suggested that a free C terminus was important in the inhibitor.

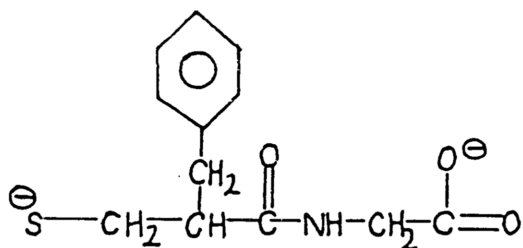
Roques et al (1980) replaced the N terminal amino group of PheGly with a sulphydryl moiety, resulting in the compound "Thiorphan", DL-3-mercapto 2-benzyl propionyl glycine. Thiorphan is a very potent and selective in vitro inhibitor of enkephalinase activity on brain membranes (Ki=3 nM) or purified "enkephalinase" (endopeptidase) from rabbit kidney (Malfroy and Schwartz, 1982); it possesses antinociceptive activity when administered to mice (Roques et al, 1980) and enhances the recovery of enkephalins released from brain slices (Patey et al 1980; De la Baume et al, 1982, 1983). Roques et al (1980, 1982) represented the binding of enkephalins or thiorphan to the active site of enkephalinase as shown in fig. 1.5.6 (c).

Subsequent work by Roques and colleagues and by Gordon et al (1983) (who developed the ACE inhibitors) has supported this model, and provided further insight into the specificity of enkephalinase.

(i) Enkephalinase possesses endopeptidase activity (section 1.5.4.) and is probably identical to the endopeptidase activities found in many body organs (section 1.5.2.a). In the model for CPA of Quiocho and Lipscomb (1971) a crucial Arg residue (145 in the sequence) binds the carboxyl terminus of the substrate; this residue



Substrate: Leucine enkephalin



Inhibitor: Thiorphan

Fig. 1.5.6(c): Model for the binding of substrate or inhibitor to enkephalinase.

would sterically hinder binding of an extended substrate. The residue Arg 203 of thermolysin at the active site would not interfere with the binding of either a carboxyl terminus or an extended peptide (Kester and Matthews, 1977); its position is slightly different so that it appears to bind the carbonyl group of the peptide bond immediately to the C terminal side of the scissile bond (Monzingo and Matthews, 1982). Malfroy and Schwartz (1982b) used butanedione to demonstrate the presence of a guanidium group in purified rat kidney "enkephalinase" and also in rat striatal membranes. The rate of hydrolysis of ME and LE was reduced by about 80%, but cleavage of endopeptidase substrates was significantly less affected. ACE is highly selective for a free C terminus and amidation of captopril reduces its potency against ACE by 80 fold, whereas enkephalinase is much less selective and amidation of thiorphan increases the IC 50 from 4 nM to 17 nM. Thermolysin has a strong preference for amidated compounds. (Roques et al, 1982). Schwartz et al (1983) point out that while in pancreatic carboxypeptidase A the binding of the carboxyl terminus is crucial to the catalytic mechanism, the lysosomal CPA does recognise amidated substrates. It seems that subtle differences in the position of the Arg residue govern this aspect of specificity.

(ii) Gordon et al (1983) re-examined the specificity of the S'1 subsite using 3 mercaptopropanoyl (3MP) amino acid derivatives. The substitution of a benzyl group at the C-2 carbon atom was optimal for the inhibitory potency of 3MPLeu, which had an IC 50 of 19 nM. This compound was the most potent of over 50 tested, and was considerably more potent than thiorphan (2 benzyl 3MP Gly) which had an IC 50 of 360 nM.

(iii) The influence of the stereochemical configuration at the C-2 carbon atom in the 3MP series of compounds was examined using isomers of 3-mercapto 2-methyl propionyl DOPA. An S,S configuration mimics that of a natural L,L dipeptide. The S,S isomer had an IC 50

of 1.34 μM versus enkephalinase and 0.15 uM versus ACE; the R's isomer (SQ 27,222) had IC 50 values of 1.43 and 0.7 μM respectively. Thus, enkephalinase is less critical than ACE of the spatial arrangement of groups bonded to the C-2 carbon atom. The compound SQ 24,994 used in the experimental work described below is racemic at the C-2 carbon atom.

(iv) Roques et al (1983) demonstrated that enkephalinase was much more tolerant than ACE of a reversed peptide bond in the inhibitor, provided the correct chirality of the R groups was maintained - for example, the S (L) form of PheGly had IC 50 3 uM; the IC 50 of the R form was greater than 100 μM, but "retro-inverso" PheGly (in which the N and C atoms of the amide bond have "changed places") in the R cinfiguration had IC 50 12 μM, while the IC 50 of the S form was over 100 μM. The compound "retro" thiorphan had an IC 50 of 6 nM versus enkephalinase (only 2-fold higher than that of thiorphan) **whereas its** IC 50 against ACE was greater than 10 μM. Since the IC 50 of thiorphan against ACE is 140 nM (Roques et al, 1980) the retro-compound is far more selective; this might be important were the compound to be tested clinically.

(v) Very potent inhibitors of metallopeptidases need not possess a sulphydryl group to ligate the zinc atom at the active site. Thus phosphoramidon, which inhibits thermolysin, neutral endopeptidase of kidney and enkephalinase of striatal membranes (Malfroy et al 1982 and Matsas et al, 1983) apparently relies on a phosphate group to bind zinc (Cushman and Ondetti 1981). Hydroxamate derivatives of peptides are potent "enkephalinase" inhibitors (Hudgin et al, 1981). Patchett et al (1980) describe carboxymethyl ACE inhibitors one of which (MK 421) has been tested clinically; this type of compound may achieve a high binding affinity by mimicry of the transition state conformation of a substrate at the active site. Fournie-Zaluski et al (1983) recently reported the synthesis of carboxylalkyl derivatives of Phe-Ala and Phe-Leu.

1.5.7. MEDICAL ASPECTS OF INHIBITORS OF ENKEPHALIN BREAKDOWN

Opiates are still indispensable for the relief of pain, particularly visceral or "deep" pain. However, opiates are liable to cause addiction; they may also produce unwelcome side effects, both peripherally (for example, reducing gut motility) and centrally (eg, depressing respiration), which arise from stimulation of opioid receptors unconnected with the pathways of pain perception. The prospect of developing an agent which would induce a morphine-like analgesia, yet be non-addictive, has motivated the development of drugs which inhibit enkephalin breakdown. Research into enkephalinase inhibitors has drawn inspiration from the successful development of inhibitors of angiotensin converting enzyme. The drug captopril is orally active (Rubin et al, 1978) and is used clinically in the treatment of hypertension (Atkinson and Robertson, 1979).

The antinociceptive effect of an inhibitor of enkephalin breakdown would presumably be mediated by an accumulation of endogenous enkephalin, as observed by Zhang et al (1982). The efficacy of such a drug in suppressing a particular algesic response might thus depend on the tonic rate of enkephalin release. It has been postulated (Roques et al, 1980; Fournie-Zaluski et al, 1983) that the opiate antagonist naloxone can only exert a pro-nociceptive (hyperalgesic) effect on those endogenous antinociceptive systems in which there is a tonic release of enkephalins. Naloxone is pro-nociceptive on the hot plate test (Frederickson et al, 1977) or the writhing response (Chaillet et al, 1983) but has no effect on the tail flick reflex (Roques et al, 1980). Similarly, thiorphan is anti-nociceptive in the hot-plate (Roques et al, 1980; Zhang et al, 1982) and writhing (Chaillet et al, 1983) models but does not prolong tail-flick latency (Roques et al, 1980). Chaillet et al (1983) consider that a noxious stimulus may have to be

prolonged, to trigger opioid release. Thiorphan potentiates stress-induced analgesia, an effect which is reversible by naloxone (Chipkin et al, 1982). Inhibitors of enkephalin breakdown might find an application in circumstances where enkephalin release might be triggered, e.g., in enhancing the effect of focal brain stimulation for the relief of pain (Akil et al 1972, 1978) or of acupuncture.

Roques et al (1980) potentiated the antinociceptive effects of D-Ala 2 ME by the co-administration of thiorphan; the same effect was obtained by Fournie-Zaluski et al (1983) using the novel enkephalinase inhibitor N - 2 - carboxy, 3 - phenyl propanoyl L - Leu. Chaillet et al, (1983) found that the antinociceptive actions of thiorphan or of bestatin, either alone or in combination, were enhanced by a 30 ug dose of ME, which had no effect without the inhibitor. Such effects are presumably attributable to the protection of the exogenous peptide by the drug. On the other hand, Hachisu et al (1982) found that a wide range of derivatives of the amino acid AHPA (section 1.3.3.), including bestatin, produced "analgesia" in the tail-flick test after systemic administration, provided that 0.5 mg/kg morphine - too small a dose to induce detectable "analgesia" - was also given. Hachisu et al speculated that "morphine analgesia results from release of an analgesic peptide". An alternative explanation is that the receptor occupancy jointly achieved by the morphine and by the accumulation of endogenous enkephalin might be antinociceptive although neither ligand alone achieved a sufficiently high concentration (as explained above, the tail-flick reflex may involve synapses at which the tonic release of enkephalin is particularly low). Conceivably, inhibitors of enkephalin breakdown might be effective as adjuvants of opiate therapy, permitting the dose of opiate drug to be reduced.

The "overshoot" of elevated enkephalinase levels following removal of a chronically implanted morphine pellet (Schwartz et al, 1980) has been considered to contribute to the

symptoms of opiate withdrawal; perhaps enkephalinase inhibitors would alleviate such symptoms.

There are thus several ways in which inhibiting enkephalin breakdown might be therapeutically useful. The initial obstacle, that of synthesizing a potent inhibitor, seems to have been overcome; thiorphan has potent *in vivo* effects in animals, while the carboxylalkyl derivatives produced by Fournie-Zaluski *et al* (1983), although less potent, might have fewer side-effects (Patchett *et al*, 1980). The latter compounds have such a low activity against angiotensin converting enzyme,as has retro-thiorphan (Roques *et al* 1983) that untoward effects on blood pressure seem unlikely. However, many potential problems remain.

(i) Inhibiting enkephalin breakdown might affect any physiological process involving enkephalins.

(ii) Because both enkephalinase and aminopeptidase are involved in the inactivation of enkephalins in the brain, (De la Baume *et al* 1982, 1983) the full protection of enkephalins might necessitate the inhibition of both classes of enzyme (Zhang *et al*, 1982; Chaillet *et al*, 1983). Bestatin protects enkephalins from amino-peptidase attack but has a broad spectrum of action; the inactivation of enkephalins throughout the body might well be undesirable.

(iii) The systemic administration of, for example, thiorphan, would probably inhibit the endopeptidases, similar (or identical) to enkephalinase, which occur in many body organs (Llorens and Schwartz, 1981). These enzymes presumably fulfil a variety of functions and their inhibition might have unpredictable effects.

(iv) The concentrations at opioid receptors of enkephalins, which would be attained by protecting them from breakdown must depend on rates of enkephalin synthesis and release. Enkephalins are capable of inducing tolerance (Brandt *et al*, 1976; Lampert *et al* 1976; Waterfield *et al*, 1977) as does morphine; tolerance to elevated

levels of endogenous enkephalins could not be circumvented simply by increasing the dose.

(v) It is also possible that enkephalin concentrations *in vivo* are homeostatically regulated; Zhang *et al* (1982) found that although thiorphan inhibited enkephalinase in the brains of mice for over 2 hours, striatal enkephalin levels peaked after 15 min. and decreased after 30 min.

1.6. PHYSIOLOGICAL IMPORTANCE OF ENKEPHALIN-DEGRADING PEPTIDASES.

That a synaptically-released agent be rapidly inactivated is a crucial requirement for its characterization as a neurotransmitter (Werman, 1966). The inactivation mechanism for enkephalins has been likened to that for acetylcholine (Schwartz et al, 1980). Six criteria have been proposed by Schwartz et al (1981) to identify an enzyme as a "neuropeptidase". Each requirement will be examined in turn :-

(i) "Enzymatic cleavage should produce biologically inactive fragments".

Any enzymatic cleavage of enkephalin will destroy its activity.

(ii) "The enzyme should be strategically located".

(a) Subcellularly :

Aminopeptidases (section 1.3.1.d).- Although only a small proportion of the total tissue aminopeptidase activity is membrane bound, one or more species of aminopeptidase might be synaptically located. Angiotensin converting enzyme - the data of Yang and Neff (1972) are consistent with the presence of this enzyme on the membranes of neurones in the brain. Dipeptidyl aminopeptidases (section 1.4) - one species (D.A.P. IV) occurs in a membrane-bound form in the kidney (Kenny et al, 1976) but data on its subcellular distribution in brain is lacking. DAP II (Gorenstein et al 1981) is located on neuronal membranes. The major fraction of "Enkephalinase B" activity is cytoplasmic (Hersh, 1982), although, as in the case of aminopeptidases, this finding cannot rule out its involvement in synaptic processes. Enkephalinase (section 1.5.1.c) - its sub-cellular distribution parallels that of opioid receptors and resembles that of the classic neurotransmitter-inactivating enzyme, acetylcholinesterase (De la Baume et al, 1980)

(b) Between brain regions.

Aminopeptidases (section 1.3.1.c); the only data available concerns

total aminopeptidase activity; once again, the association of a subpopulation of aminopeptidase with enkephalins cannot be ruled out. Angiotensin converting enzyme - its regional distribution is distinctly different from those of opioids or their receptors (Swerts et al 1979 b; Schwartz et al 1980).

Dipeptidyl aminopeptidases - information is sketchy. DAP II has a distribution so selective, and so unlike that of opioids that it must surely be ruled out. (Gorenstein et al, 1981). "Enkephalinase B" is uniformly distributed throughout the brain (Gorenstein and Snyder, 1980). Enkephalinase - uniquely among the enzymes described here, enkephalinase has a distribution similar to opioid receptors and enkephalins (section 1.5.2.d), although its regional variations are less pronounced. This is circumstantial evidence of an involvement of the enzyme with enkephalinergic transmission.

(iii) "Substrate specificity should account for the increased biological activity of synthetic analogues".

The search for better analgesics has led to the preparation of numerous peptide analogues of enkephalins. Replacement of Gly 2 by D-Ala 2 increases antinociceptive activity by three-fold; this modification confers resistance to aminopeptidase attack (Roemer and Pless, 1979) and reduces the affinity of enkephalinase for the peptide by about three-fold. Methylation of the Gly 3-Phe 4 bond of ME reduces affinity for enkephalinase by about fifteen times, and increases the antinociceptive activity twelve times (Fournie-Zaluski et al, 1979); this modification would not be expected to have much effect on recognition by aminopeptidases. More extensive modifications, impairing recognition by both aminopeptidases and enkephalinase, e.g., in D-Ala 2, Me amide (Pert et al, 1976) or in Tyr-D-Ala Gly (Methyl) Phe Met(o)ol (i.e. Sandoz FK 33-824; Roemer et al, 1977) increase the antinociceptive effect

to a much greater degree than that expected on the basis of the individual modifications (these improvements in potency are to a lesser extent attributable to an enhanced affinity for the receptor). That the Sandoz peptide has an antinociceptive potency 30,000 times that of ME when given i.c.v. demonstrates that both enkephalinase and aminopeptidases limit the access to opioid receptors of enkephalins administered i.c.v. but is only circumstantial evidence that synaptically released opioids are inactivated by these enzymes.

(iv) "Enzyme activity might reflect adaptive changes to modified neurotransmission".

The implantation into mice of a morphine pellet for two days is followed by a progressive increase in their striatal enkephalinase activity; after four days, enkephalinase reaches a peak at about 125% control level, although the implant was removed on the second day. Activity falls sharply on the fifth day (Schwartz et al, 1980). Protracted high occupancy of opioid receptors may initiate a long-term homeostatic process, which may in turn diminish the concentration at the receptor of the endogenous opioid. There was no measurable effect on the activities of aminopeptidases and angiotensin converting enzyme. Narcotic receptor binding is unaffected by morphine dependence (Klee and Streaty, 1974). Neither are brain enkephalin levels affected (Bonnet et al, 1976; Childers et al, 1977). Thus, enkephalinase may be involved in mechanisms of opiate withdrawal (Schwartz et al, 1980). The experiment at least suggests a particular involvement of enkephalinase in physiological inactivation of enkephalins, although the findings do not preclude the participation of a sub-population of amino-peptidases.

(v) "Enzyme inhibition should protect synaptically-released neuropeptides".

Thiorphan will selectively inhibit enkephalinase but not aminopeptidase or angiotensin converting enzyme, and will double the recovery of enkephalins released from striatal slices in vitro by high potassium ion concentrations (Patey et al, 1981). A subpopulation of aminopeptidase contributes to enkephalin degradation in this model, since the aminopeptidase inhibitor, bestatin, also enhances recovery, although puromycin - which inhibits the breakdown of exogenous enkephalins - will not. The potent ACE inhibitor, captopril, has virtually no effect. A combination of bestatin and thiorphan almost completely protects the released enkephalin (De la Baume et al, 1982, 1983; Chaillet et al, 1983). These results suggest that the localization of the enzymes in crucial; but it is not possible to assess the exact relative contributions of enkephalinase and aminopeptidase in vivo, since the enkephalins are assayed only after they have diffused into the medium bathing the slices, and en route they may still encounter enzymes which are not of primary importance in terminating the enkephalinergic "message". However, Zhang et al (1982) reported that thiorphan i.c.v. increased striatal ME content in mice, and that bestatin with thiorphan produced a significant further increase, although bestatin alone had no effect. Chaillet et al (1983) found that ME introduced i.c.v. into mice in vivo was protected by bestatin or thiorphan or both, but not by puromycin.

(vi) "Enzyme inhibition reproduces the biological activity of the neuropeptide".

The effects of enzyme inhibitors on nociception in mice have been studied by Zhang et al (1982), in parallel with the enkephalin content of the striatum (see above); thiorphan was significantly antinociceptive, and its action was potentiated by bestatin, which, however, had no significant effect alone.

Chaillet et al (1983) and De la Baume

et al (1983), on the other hand, reported that bestatin given alone was antinociceptive in the same animal model (hot plate jump test) and at a lower dose (50 µg instead of 75 µg). These workers found that the effects of thiorphan were similar to those described by Zhang *et al* (1982).

Summary.

Together with the results on *in vivo* enkephalin content, the effects of drugs on nociception indicate that enkephalinase is involved in this physiological system, while a sub-set of membrane-bound aminopeptidases are also involved; these conclusions are consistent with the results obtained using the striatal slice model. It is now apparent that the criterion (ii), and the lack of effect of puromycin, initially led workers in this field to the false conclusion that aminopeptidases were not of physiological importance in enkephalin inactivation. In fact, the multiplicity of aminopeptidases capable of degrading enkephalins *in vitro* means that distribution studies using this substrate are insufficiently specific. For the same reason, it is impossible to be certain that a specific class of aminopeptidases is not affected by morphine implantation (criterion (iv)).

There is no evidence at present to suggest that dipeptidyl aminopeptidases such as "enkephalinase B" are involved in enkephalin breakdown. The consensus view is that the angiotensin converting enzyme is not physiologically important in degrading enkephalins; if this is the case, then the analgesic effects of captopril reported by some workers (Ercan *et al*, 1980; Türker *et al* 1983) cannot be produced by an inhibition of ACE **activity.** It has been suggested (Zhang *et al*, 1983) that angiotensin converting enzyme generates ME from ME-Arg 6-Phe 7, and that accumulation of the heptapeptide in the presence of captopril has an antinociceptive effect. The function of this enzyme in the

brain is controversial (Brownfield *et al*, 1972).

1.7 AIMS OF THE PRESENT STUDY

Several of the enzymes which may be responsible for the physiological degradation of enkephalins have now been characterized *in vitro* in some detail. However, far less is known of the function of these enzymes in the intact animal; the parts played by the various alternative catabolic pathways are not clear. Experiments on the anti-nociceptive effects of enzyme inhibitors have yielded somewhat ambiguous results. The results obtained by other groups of workers using the two main *in vitro* models - either washed striatal membranes or viable striatal slices - have also been inconsistent. In the experiments described below, these two systems will be examined in parallel, enabling some conclusions to be drawn about their relative suitability as models of the physiological situation. To clarify the extent of enkephalin breakdown by each of the possible pathways, techniques of purification will be described which permit the quantification in every experimental incubation of all four primary products of the enzymic cleavage of $^{3}H[Tyr]^{1}$ LE substrate. The Km values for both "enkephalinase" and amino-peptidase are of the order of tens of micromolar, yet the Km values of enkephalins at opioid receptors are at least two orders of magnitude lower than this. It appears desirable, therefore, that the concentration of enkephalin in the *in vitro* experiments should test the ability of the enzymes to reduce the concentration of enkephalins to levels at which their receptor occupancy ought to be low; a substrate concentration of 16 nM LE was therefore chosen. It was considered valuable to assay the enzyme activities of the striatal membrane preparation in a "physiological" inorganic saline, rather than the Tris or similar organic buffer used by other workers, enabling a more direct comparison to be made with the results obtained using striatal slices. Peptidase activity

may be affected by the binding of metal cations at sites other than the active site - for example, leucine aminopeptidase is activated by divalent Mg or Mn ions (Carpenter and Vahl, 1973) while some endopeptidases autolyse in the absence of Ca ions (Cushman and Ondetti, 1981).

In the following experiments, considerable use will be made of three inhibitors of enkephalin breakdown - thiorphan (Rogues et al., 1980), bestatin (Barclay and Phillips, 1980) and puromycin (Vogel and Altstein, 1978). The selectivity of their effects will first be evaluated before going on to consider their usefulness as experimental tools for the isolation of the activity of a single enzyme type in a mixture of peptidases such as those found in a striatal slice or membrane preparation. In addition the compound SQ24994, designed as a novel "enkephalinase" inhibitor, will be compared to the well-characterized compound, thiorphan.

Results published by Harsing et al. (1982) implied that an "enkephalinase inhibitor" the dipeptide Phe-Ala could increase the intracellular enkephalin content of superfused striatal slices in vitro. This finding suggested that there might be an intracellular enzyme activity which was sensitive to the same types of inhibitor as "enkephalinase" and raised the intriguing possibility of the existence of intra-neuronal peptide turnover. It has been stressed above (section 1.3.1) that enkaphalin-degrading aminopeptidases are found both in the cytoplasm and bound to membranes; it is conceivable that aminopeptidase activity might be capable of degrading enkephalins within neurones.

Experiments will be described below, in which viable striatal slices were superfused with media containing bestatin, SQ24994 or both, and the endogenous ME and LE were chromatographically purified, before quantitation by means of an enzyme-linked immunoabsorbent assay (Zamboni et al., 1983).

An enzyme studied in vitro cannot of course be implicated in physiological processes unless its effects can be demonstrated in a relevant in vivo model. An established behavioural paradigm for assessing opioid activity is the demonstration that a substance can attenuate the response of an intact animal to noxious stimuli. An inhibitor of enkephalin breakdown might increase the enkephalin concentrations at receptors, thereby exerting such an antinociceptive effect. Roques et al. (1981) had already reported on the antinociceptive activity of thiorphan. Following the demonstration of its antinociceptive properties (P. M. Caesar, unpublished data), SQ24994 was administered to rats by intra-cerebra ventricular injection in vivo; its effects on the levels of ME and LE in the brain will be described below.

Isolated organ preparations from the gut, the vasculature or the urinogenital system were important tools in the identification of different classes of opioid receptor, and the mouse vas deferens, a sensitive bioassay for δ-agonists was used by Hughes (1975) during the first purification of the enkephalins. Compared to preparations from the CNS, the bioassay can have useful practical advantages, such as robustness and an instantaneously measurable mechanical response. Data will be presented below, demonstrating an enhanced sensitivity of the mouse vas to LE in the presence of SQ24994; these results are circumstantial evidence that an SQ24994-sensitive enzyme can limit the concentration of exogenously applied enkephalin at these peripheral δ receptors.

M A T E R I A L S

2. MATERIALS.

RADIOLABELLED PEPTIDES.

^3H - Tyr 1 LE	: Amersham International or New England Nuclear.
^3H - Tyr	: Amersham International.

STANDARD SYNTHETIC PEPTIDES.

LE; ME; Tyr Gly Gly; Tyr Gly; Tyr	: Sigma.
Tyr Gly Gly Phe	: Cambridge Research Biochemicals.

DRUGS.

Bestatin, Puromycin	: Sigma.
SQ 24,994; Thiorphan	: Gifts from the Squibb Institute, New Jersey, to Prof. J. Hughes.

RESINS FOR CHROMATOGRAPHY.

Sephadex G 10, G 25	: Pharmacia.
XAD2 hydrophobic resin	: Amberlite.
Porapak.Q hydrophobic resin	: Waters.
S.M.7; S.X.2	: Bio-Rad.
Co-Pell ODS C-18	: Whatman.
SepPak C.18 (disposable cartridges)	: HPLC technology.
CG.400 anion exchanger	: Amberlite.
Dowex 50.W cation exchanger	: BDH.

THIN-LAYER CHROMATOGRAPHY PLATES.

LK5.D Silica, channeled plates: Whatman.

SOLVENTS.

Tri-fluoracetic acid, "special spectroscopy"	: Sigma.

All other organic solvents were "AnalaR" grade, obtained from Sigma, BDH or Fisons.
Inorganic salts, acids, bases and standard laboratory reagents were AnalaR quality, from the usual suppliers.

SCINTILLATION COUNTING.

PPO; Dimethyl POPOP	: Sigma.
Triton X - 100	: Rohm and Haas.

GLASSWARE.

All glassware which was to come into contact with peptides was treated with "Sigmacote" (Sigma) according to the manufacturer's instructions, and washed with dilute HCl and detergent solutions after treatment. The Sigmacote produces an inert coating which avoids the loss of peptides due to adsorption to glass surfaces.

ANIMALS.

Sprague-Dawley rats and T/O strain mice were bred at Imperial College.

M E T H O D S

3.1. PREPARATION OF CORPUS STRIATUM SLICES.

An adult male Sprague-Dawley rat, of approximate body weight 250 g, was killed and its brain removed. The brain was placed on an ice-cooled glass Petri dish, while the corpora striata were dissected out.

Slices, of nominal thickness 350 μm, were cut using a Campden Instruments "Vibroslice". The tissue to be cut was blotted gently and fastened to the Teflon stage using "Superglue" (Cyano-acrylate adhesive). The Teflon block was mounted on the instrument within a bath containing Krebs-Ringer solution at $4^{o}C$, gassed with a 95% O_2/5% CO_2 mixture; the tissue was immersed in physiological buffer solution throughout the slicing procedure. Both corpora striata from one rat were attached to the block side by side, and were sliced simultaneously. The tissues were cut in the sagittal plane, which is less likely to disrupt the neuronal organization of the tissue.

The bath-block assembly was transported by a manual rack-and-pinion device towards a single-edged razor blade mounted horizontally in a clamp which was electrically vibrated at a high frequency. After cutting each slice, the blade was lowered by a second rack-and-pinion coupled to a Vernier scale, thus regulating the slice thickness. Once cut, a slice was retrieved from the bath by means of a drawn-out Pasteur pipette, and transferred to ice-cold, gassed Krebs-Ringer solution. About 15 min. elapsed between the death of the rat and the completion of slicing. It two animals were used, the tissue slices from the first rat were stored at $4^{o}C$ while slices were prepared from the second; the slices were then pooled.

3.2. SUPERFUSION OF CORPUS STRIATUM SLICES.

The superfusion chamber was constructed from the barrel of a disposable syringe, fitted with plungers at both ends. During the experiment, the syringe barrel was submerged in a water bath at 37° C. The rubber piston at the lower end was pierced by two tubes, one of which carried the inflow of pre-gassed Krebs-Ringer solution at 37° C; the other was used to gently bubble the contents of the chamber with 95% O_2/5% CO_2. Through the uppermost piston passed a short tube which acted as a sleeve for the outflow tube; since the outflow was a loose fit in this sleeve, the chamber was not airtight. The volume of liquid in the superfusion chamber was regulated by the level of the outflow opening. To exclude the tissue slices the open end of the outflow was covered with fine nylon gauze. Both the inflow and the outflow were pumped by peristaltic pumps; however, the outflow was always pumped at a very high rate, so that the flow rate was determined by the inflow pump.

The Krebs-Ringer medium used for superfusion was that described by de Belleroche and Bradford (1972) and was of the following composition (mM):
NaCl, 121; KCl, 2; KH_2PO_4, 1.25; $MgSO_4$, 1.31; $NaHCO_3$, 26; $CaCl_2$, 2.6; Glucose, 10. This solution was used for all the experiments using striatal slices, and for the studies on LE breakdown using a striatal membrane preparation.

3.3 (a) PREPARATION OF MEMBRANES FROM CORPUS STRIATUM.

(i) Media.

The preparation was based on those described by Fournie-Zaluski et al (1979) and Malfroy et al (1979) in which a Tris. HCl buffer was used as the homogenization medium. However, the Tris. HCl was replaced with Krebs-Ringer solution in order that the ionic conditions should be exactly the same as those in the brain slice incubations, which were probably closer to those found in brain tissue in vivo. It was then also possible to use exactly the same purification methods for the enkephalin metabolites from both types of tissue preparation.

(ii) Tissue source.

Throughout the metabolism experiments, corpora striata from Sprague-Dawley rats of body weight 200 - 250 g were used.

(iii) Homogenization.

A Jencons "Ultra-Turrax" motorized homogenizer was used at half speed for 15 sec to homogenize one pair of corpora striata into 50 ml ice-cold, 50 mM Tris HCL buffer (pH 7.4).

(iv) Centrifugation and washing.

Debris was pelleted by a preliminary centrifugation at 1000g for 5 min. The supernatant was centrifuged at 12000g for 15 min, the supernatant discarded and the surface of the pellet washed with 5 ml Tris. HCL buffer before resuspension by means of the homogenizer into 50 ml Tris. HCL. The membranes were re-pelleted by centrifugation at 12000g for 15 min. This washing procedure was repeated four times before resuspension in 50 ml Krebs-Ringer solution (section 3.2). After a further washing step with Krebs-Ringer solution the membranes were resuspended in 1.5 ml Krebs-Ringer medium for use in the experiments. When an experiment required more than 1.5 ml of membrane suspension, separate 1.5 ml preparations were pooled at this stage.

The preparation was generally stored on ice for one to two hours before the experiment; it was thoroughly mixed at intervals to prevent settling. Before use, the preparation was brought to experimental temperature (37°C) in a water bath.

3. (b) METABOLISM OF LE BY BRAIN MEMBRANES.

The preparation of corpus striatum membranes was incubated with ^{3}HLE as substrate; the products of LE metabolism were separated and quantitated.* A comparison was made of the effects on LE breakdown of the compounds Puromycin, Bestatin (each at concentrations of 100nM, 1μM, 10μM, 100μM and 1mM) Thiorphan and SQ 24994 (each at 100pM, 1nM, 10nM, 100nM, 1μM and 10μM) and of combinations of 100uM Bestatin and either Thiorphan or SQ 24,994 (each of the latter was tested over the range 100pM to 10μM).

Drug solutions were prepared in Krebs-Ringer buffer at 5 times the desired final concentration. ^{3}HLE was made up in the same way, using tracer freshly purified by HPLC (section 3.5). The final concentration of ^{3}HLE in the incubation mixture was 8.08 nM. (section 1.7).

Experimental Incubations.

The reaction vessels were 1.5ml micro centrifuge tubes; all incubations were at 37°C.

In every experiment one of the tubes contained only 400μl Krebs-Ringer buffer plus 100μl of the tracer solution, but was otherwise processed identically to the tubes containing membrane preparation. This "no tissue" incubation controlled against non-enzymatic degradation of the LE, and provided an estimate of background radioactivity arising during TLC. (See section 4.3.2.).

(i) 300μl membrane preparation was pre-incubated at 37°C with either 100μl Krebs-Ringer solution alone or with 100μl of the solution of a test compound.

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*See sections 3.6, 3.7 and 3.8.

(ii) 15 min. after addition of drug to a tube, the ^{3}HLE substrate was added. The tracer was incubated with the membrane suspension for 15 min; during this period the tubes were mixed frequently.
(iii) At the end of the 15 min, enzyme action was stopped by adding 25µl, 1 M HCL. After mixing the tube was transferred to ice. (In a preliminary trial of this method HCL was added immediately following the addition of the ^{3}HLE - the composition of the sample was found by TLC to be identical to that of the no tissue incubation).
(iv) To remove the brain membranes from the reaction mixture the tubes were centrifuged at 12,000 x g for 15 min in an Eppendorf bench centrifuge at 4°C. 450µl of supernatant was removed from each tube. Unless Dowex chromatography followed immediately the samples of supernatant were rapidly frozen by means of a mixture of solid CO_2 and propanol and then stored at -20°C.

3.4 METABOLISM OF ^{3}H LE BY CORPUS STRIATUM SLICES.

Viable slices of rat corpus striatum were incubated with radioactively labelled LE and the products of its metabolism measured using TLC. The compounds SQ 24,994 and Bestatin, the effects of which had been studied using a membrane preparation from the same tissue source, were introduced into the system and their effects on LE metabolism were studied.

(a) Preparation of Slices.

See section 3.1; slices were prepared from one Sprague Dawley rat of about 250g body weight.

(b) Drug and Substrate Solutions.

The compounds used were dissolved in Krebs-Ringer solution such that the addition of 50µl of the same buffer without drug to 950µl of the drug solution would give the desired final concentration. Tritium-labelled LE, freshly purified by HPLC, was made up in Krebs-Ringer buffer so that 50µl of the solution contained 8.08 pmol LE; when 50µl of this LE solution was added to 950µl Krebs solution the final LE concentration was then 8.08 nM.

(c) Pre-incubation of Slices.

After their preparation the slices were superfused as described for one hour (section 3.2) in a volume of 10ml and with a flow rate of 1.0ml/min, to remove debris arising from slicing and to allow the tissue to attain a stable condition.

(d) Incubation of Slices with ^{3}H LE.

The incubations were preformed in the wells of a "Falcon" disposable tissue culture plate. The lid of the plate was pierced above each well, allowing a 25-gauge syringe needle to pass through, that the solution in the well might be gassed with the 95% O_2/5% CO_2 mixture. The plate was fastened to the trolley of a shaking water bath, with the base of the plate just below the surface of the water in the bath; the plate was shaken gently throughout the incubations. The

thermostat was adjusted so that the liquid in the wells of the plate was at 37°C.

In each experiment LE was incubated under six different conditions.

1) With striatal slices, in the absence of drug - "CONTROL"
2) With striatal slices, in the presence of 100µM Bestatin
3) With striatal slices, in the presence of 10µM SQ 24,994
4) With striatal slices, in the presence of 10µM SQ 24,994 plus 100µM Bestatin
5) In Krebs solution which had previously contained tissue slices - the "TISSUE REMOVED" incubation.
6) In Krebs solution alone - the "NO TISSUE" incubation

The experiment was replicated seven times.

After 1 h superfusion the slices were emptied into a Petri dish containing gassed Krebs solution at 37°C and then distributed as in equal numbers between four wells of the plate. Each of these wells was then filled with 50µl Krebs-Ringer solution or one of the three drug solutions; the slices were incubated thus for 15 min.

The Krebs solution containing no drugs, which had been put in one well, was transferred to another, empty well. 950µl fresh Krebs was added to the slices; this latter was used as the "CONTROL" incubation. The solution which had been in contact with the tissue slices served as a control for enzyme activity leaking from the slices into the medium - the **"TISSUE REMOVED"** incubation. The drug solutions in the other wells were removed, discarded and each replaced with 950µl of the relevant drug solution. A sixth, empty well received 950µl fresh Krebs-Ringer solution as the "NO TISSUE" incubation - a control for radioactivity appearing on the TLC plate in the absence of enzymatic activity. To allow time for these manipulations, the wells were dealt with in turn at intervals of 30 sec; this timing was adhered to throughout the experiment so that all the incubations lasted for

the same time. To start the reaction, 50µl ^{3}HLE solution was added to each well in turn. After 15 min. incubation, 800µl of the reaction mixture was removed from each well and added to 50µl, 5 M HCL to stop enzyme activity. The samples were each rapidly frozen in a mixture of solid CO_2 and propanol, and were stored at -20°C until required for Dowex 50-W chromatography.

After the experiment, the tissue slices from each well were separately dispersed into 1ml, 2M NaOH using an ultrasonic probe. These samples were stored at -20°C and later assayed for protein using the Lowry method (section 3.18).

3.5. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

Equipment:

KONTRON LIQUID CHROMATOGRAPHY PUMP, MODE L.C.10

TECHSPHERE 5-ODS, C-18 COLUMN

PYE UNICAM ULTRAVIOLET DETECTOR, MODEL LC3

RHEODYNE INJECTION HEAD, CAPACITY 200 ml.

VITATRON FLAT-BED CHART RECORDER.

Elution conditions:

SOLVENT: 75 ml. METHANOL AR; 25 ml. 0.08% AQUEOUS TRIFLUOROACETIC ACID.

FLOW RATE: 0.6 ml./min.

PRESSURE: 80 BAR

ULTRAVIOLET ABSORBANCE WAS MONITORED AT 210 nm.

After use, pure methanol was run through the column at a flow rate of 1.5 ml./min.; the cleaned column was stored in methanol. Subsequently the column was re-equilibrated in the elution solvent which was pumped through at a flow rate of 1.0 to 1.5 ml./min. for 2 or more h., before use for sample purification. Solvents were always de-gassed before use.

Purification of ^{3}H LE by HPLC.

Radiolabelled LE deteriorated during storage at 4°C, under the conditions recommended by the manufacturer. The u.v. trace obtained by HPLC of a fresh stock of ^{3}H LE(Amersham) is shown in figure 3.5(a). Figure 3.5(b) shows the same solution after 4 months storage at 4°C. The chromatography conditions are described above. It was therefore essential to purify labelled LE routinely, before its use as substrate in the metabolism experiments (sections 3.3 and 3.4).

The column was calibrated by running about 1 ug authentic unlabelled LE. The eluate from a sample of the radioactive peptide was collected by hand in 1 min. fractions. A 5 ul portion of every fraction was scintillation counted (section 3.8) and fractions containing the peak of tritium counts, coincident with the elution time of standard LE, were pooled together and rotary-evaporated to dryness. The peptide was stored dry at -20°C for up to 1 week before use.

A preliminary purification by TLC (section 3.6b) yielded a tracer of higher purity, judged by HPLC analysis. However the recovery from TLC is poor and the improvement produced

by the combined procedure was considered too slight to make its routine use worthwhile.

Separation of LE catabolites by HPLC.

Four of the catabolites of LE contain tyrosine, and can be quantified by scintillation counting if ^{3}H Tyr - LE is used as substrate; the separation of these four substances was attempted, using HPLC. The results are presented in section 4.2 .

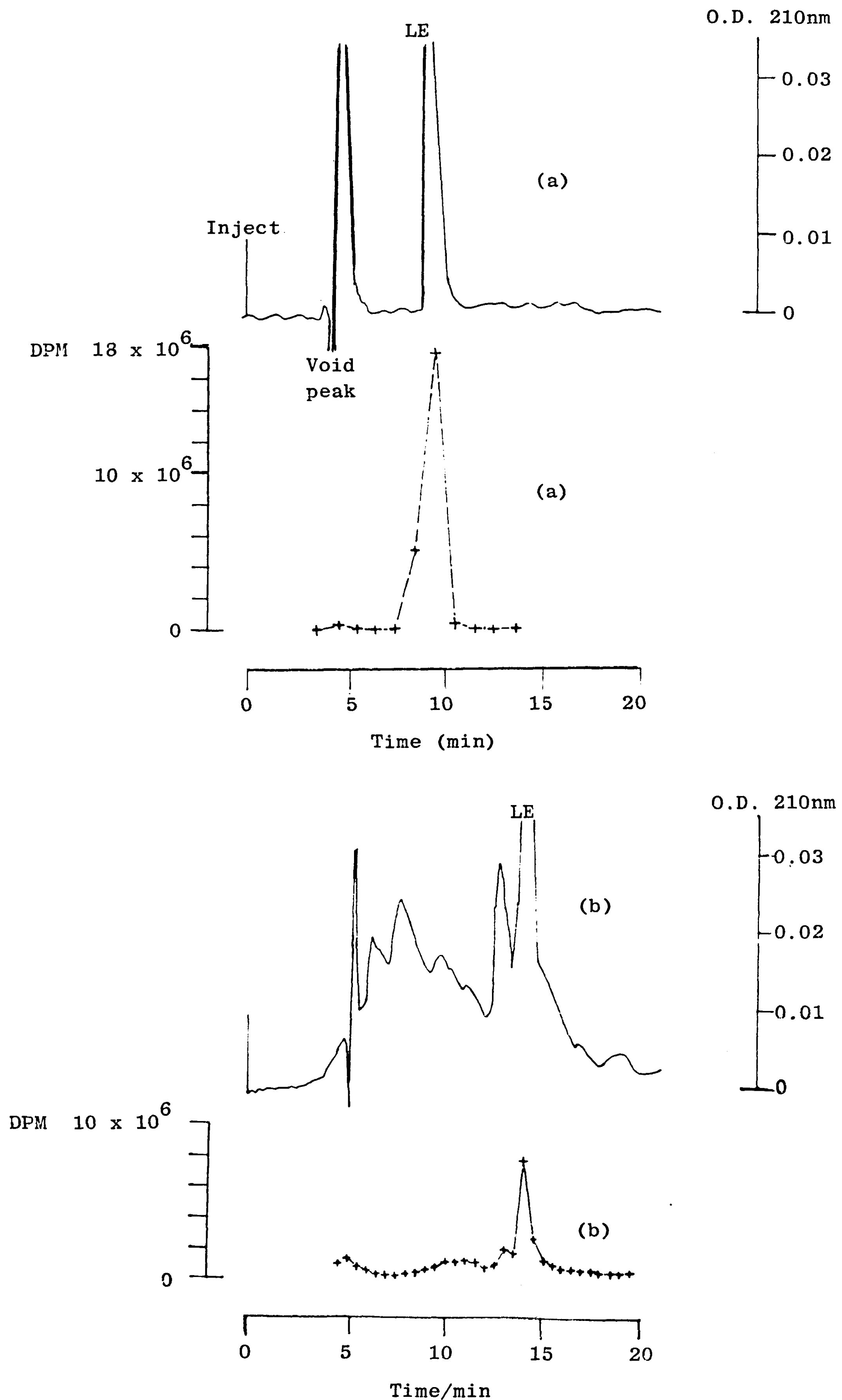

Fig. 3.5: Purification of ^{3}H LE by high performance liquid chromatography. (a) Fresh stock; (b) After 4 months storage at $4^{o}C$.

3.6 THIN LAYER CHROMATOGRAPHY.

(a) Preliminary trials.

A wide range of solvent systems was tested, using Whatman LK 5 D channeled silica gel plates. To simulate the composition of the samples from the degradation experiments, the standard solution contained 1 μg each Tyr, Tyr-Gly, Tyr-Gly-Gly, Tyr-Gly-Gly-Phe per 200 μl of a Krebs-Ringer solution containing 5% HCl (conc).

Plates were developed until the solvent front had migrated 13 to 15cm from the origin. After drying the plates, the peptides were detected by spraying with ninhydrin (1% w/v) in acetone. The following solvent mixtures were tested initially.

(a) Propan 2 ol .... 5 parts
3% NH_3 (aq) .... 4 parts

(b) Methanol .... 2 parts
Ethyl Acetate .... 2 parts
5% Acetic acid (aq) .... 1 part

(c) Ethyl Acetate ....100 parts
Pyridine .... 43 parts
Water .... 25 parts
Acetic acid .... 11 parts
(McKnight et al, 1979)

(d) Butan 1 ol .... 15 parts
Acetic acid .... 3 parts
Water .... 12 parts
Pyridine .... 10 parts

(e) Propanol .... 11 parts
Pyridine .... 4 parts
Acetic acid .... 3 parts
Water .... 1 part
Ethyl Acetate .... 11 parts
(Brenner et al, 1969)

(f) Propan 2 ol .... 2 parts
Ethyl Acetate .... 2 parts
5% Acetic acid (aq) .... 1 part

(Malfroy et al, 1979)

Only mixtures (e) and (f) separated the standards and of these (f) was much superior. Attempts to further improve the resolution by altering the relative amounts of the constituents of (f) were not successful.

It was found that the presence of the salts from the Krebs-Ringer solution was deleterious to the TLC separation; all samples were therefore de-salted before TLC analysis (section 3.7 c).

(b) Final method.

The procedure below was used for the experiments on LE breakdown by both corpus striatum slices and striatal membranes.

Sample application :-

After de-salting (section 3. 7 c) the dried samples were dissolved in 200 μl methanol and stored on ice. To each sample was added 10 μl of an aqeous solution containing 0.5μg each of LE, Tyr-Gly-Gly-Phe, Tyr-Gly-Gly, Tyr-Gly and Tyr, per 10 μl. The samples were streaked onto the pre-adsorbent areaof the plate in several applications, each of approx. 30 μl. Development and staining:- the plates were developed in a tank containing solvent (f) (see above) to a depth of about 0.5 cm. When the solvent front had migrated approx. 15 cm from the origin, the plates were removed and dried. The plates were stained by spraying with fluorescamine, (0.01% w/v in acetone). After drying for 20 min at room temperature, the bands were visualized under u.v. light.

Recovery of sample radioactivity:- each zone corresponding to a peptide standard was scraped off the glass with a small scalpel, and the silica powder sucked into a 1 ml disposable pipette tip plugged with cotton wool. The powdered silica and the cotton wool were transferred to a 5-ml "mini vial" for counting in 4.5 ml scintillation

cocktail. Counting efficiency, estimated by internal standardization, was found to vary slightly from sample to sample, although it was not related to the quantity of silica counted. Efficiency was therefore estimated using the "external standards ratio" for every sample (section 3.8)

data presented in tables 4.1 a, b and c, the following procedure was adopted.

(i) Samples were diluted with water to 5 ml, adjusted to pH 1.7 with HCl, and poured onto the column.

(ii) The column bed was washed with 5 ml HCl at pH 1.7.

(iii) 25 ml distilled water was passed through the column.

(iv) The sample was eluted with 5 ml, 10% NH_3 (aqeous).

The entire chromatography procedure was carried out at 5° C.
Eluates were rotary-evaporated and stored dry at -20° C.

3.7 DE-SALTING PROCEDURES.

Inorganic salts in the sample adversely affected TLC separation. Since it was desirable to perform the experiments in a "physiological" buffer system, methods were sought for the removal of these salts.

(a) Solvent extraction.

Samples were rotary-evaporated and the dried residue then extracted with either propan-2-ol or ethyl acetate. Although this procedure appeared to elimenate salts, the recovery of tritiated material was both low and extremely variable.

(b) Hydrophobic interaction chromatography.

Various hydrophobic resins were packed into small (2 cm height, 1 cm diameter) columns. Samples containing standard peptides were applied in Krebs-Ringer solution, the columns washed with water, and elution attempted using methanol, ethanol or propanol. Several materials (Amberlite XAD.2; Porapak Q; Biobeads SM 7 or SX 2; Whatman Co-Pell ODS; Sep-Pak C 18) retained LE and Tyr-Gly-Gly-Phe which could be eluted with the organic solvent. However, none of these resins bound free Tyr.

(c) Ion exchange chromatography.

The strongly acidic cation exchanger, Dowex 50-W, was an effective means of removing salts.

The resin was pre-cycled in bulk, with three cycles alternating washes of 20 volumes each of 1M NaOH, distilled water and 1M HCl. Before use, the gel was washed with water until the pH rose to pH 1.7 (the pH used for sample loading), and was then poured into 0.6 cm diameter, LKB "Econo-columns", to a bed height of 1.5 cm. Each column was washed with 30 ml HCl at pH 1.7 (approx. 20 mM) before applying the sample.

For calibration, standards, containing 30 pmol each tritiated LE and Tyr (purified by HPLC), were made up in Krebs-Ringer buffer to mimic the conditions of sample application. On the basis of the

3.8 SCINTILLATION COUNTING.

The scintillation cocktail was of the following composition:-

TOLUENE	3.33	litres
TRITON X 100	1.67	litres
PPO	24.2	g
DIMETHYL POPOP	750	mg

Up to 10% (v/v) of aqeous sample could be counted without efficiency falling below 25% provided that the sample contained no quenching agents. In the absence of water, standard ^3H hexadecane yielded efficiencies approaching 35%.

A Kontron Intertechnique counter was used, either with a conventional tritium counting window (in which case efficiencies were determined by internal standardization with ^3H hexadecane) or with a special programme providing an "external standards ratio" (ESR). To calibrate the instrument for the ESR, 80 vials containing 20 µl ^3H hexadecane (68,600 dpm) and various amounts of water up to 500 µl were counted. A graph was plotted of counting efficiency versus ESR.

3.9 DEGRADATION OF ^{3}H-LABELLED LE : CALCULATION OF RESULTS AND STATISTICAL ANALYSIS .

When a sample of the medium in which radio-labelled LE had been incubated with striatal slices or membranes , was purified by thin-layer chromatography , the total amount of radioactivity ("dpmt") recovered fromthe relevant track on the TLC plate was related to the quantity of LE originally present in the incubation medium :

ORIGINAL QUANTITY OF LE = TOTAL dpm UNDER ALL 5 STANDARD SPOTS (dpmt)
÷ OVERALL % RECOVERY (Rt)
÷ SPECIFIC ACTIVITY (Sa)

Similarly , for any particular product "P"

QUANTITY OF LE CONVERTED TO P = dpm UNDER STANDARD SPOT FOR THAT PRODUCT (dpmp)
÷ % RECOVERY OF P (Rp)
÷ SPECIFIC ACTIVITY (Sa)

$$\therefore \text{QUANTITY OF LE CONVERTED TO P} = \frac{dpmp}{dpmt} \times \frac{Rt}{Rp} \times \frac{Sa}{Sa} \times \text{ORIGINAL QUANTITY LE}$$

Because all the radioactivity originated from the labelled Tyr of the LE , the specific activity terms cancel . Evidence will be presented in section 4.3.2 to justify the assumption that Rt = Rp:

$$\therefore \text{QUANTITY OF LE CONVERTED TO P} = \frac{dpmp}{dpmt} \times \text{ORIGINAL QUANTITY OF LE .}$$

When any peptide is chromatographed on a thin-layer plate , most of the material is concentrated in a zone or spot . However , a small proportion is left behind as a tail , running back from the spot to the origin . Thus when several substances are chromatographed together , a small quantity of material from the fastest moving spot will contaminate the slower-migrating components of the mixture . A partial correction for this streaking effect was based on the distribution of radioactivity along the track of a sample of purified ^{3}H LE (the "NO TISSUE" sample) which had not come into contact with the brain membranes or slices , but which had otherwise been treated in exactly the same way as any of the experimental samples . Suppose the radioactivity, arising solely from pure LE , in a zone of Rf characteristic of a particular breakdown product was "NO TISSUE dpmp" , while the total radioactivity recovered from all zones of the plate was "NO TISSUE dpmt" . Based on evidence described in section 4.3.3. , the amount of "background" radioactivity

contributed to each zone of a TLC track was considered to be proportional to the quantity of LE left unchanged at the end of the incubation period ("SAMPLE dpmLE")

$$\text{CORRECTED BACKGROUND dpm} = \frac{\text{NO TISSUE dpmp}}{\text{NO TISSUE dpmt}} \times \text{SAMPLE}$$

Then

$$\text{QUANTITY OF LE CONVERTED TO P} = (\text{dpmp} - \text{CORRECTED BACKGROUND dpm}) \div \text{dpmt} \times \text{ORIGINAL QUANTITY OF LE}.$$

The rate of reaction was expressed as the final quantity (in fmol) of product formed, averaged over the entire 15 min. incubation period. This rate (in fmol/min.) was normalized for the quantity of membrane suspension present in the individual reaction vial, determined as mg of protein by the Lowry procedure. Thus the units of reaction rate were fmol/mg/min. The effects of treatments were assessed by means of a single factor analysis of variance ("anovar"). The data for LE or for any one of the products was in turn regarded as the "population" and each drug concentration constituted a "treatment". The statistic, "F" was employed initially to compare the variation between all treatments with that attributable to random variation ("residual variation"); if justified, further analysis compared individual treatments in pairs, again by an F test - statistically equivalent to Student's "t" but with the advantage of using the population residual variation as denominator.

3.10 VARIATION WITH TIME OF THE ENKEPHALIN CONTENT OF SUPERFUSED CORPUS STRIATUM SLICES.

A study of the loss by metabolism (or possibly gain by synthesis) of enkephalins in superfused slices was considered helpful to the interpretation of the possible effects on endogenous enkephalin content (section 3.10) of drugs used to inhibit the metabolism of exogenous enkephalin.

Corpus striatum slices were either homogenized immediately ("zero time") or superfused for 15 or 60 min before extraction. The superfusion chamber was of 10 ml volume and the flow rate was 1 ml/min. Throughout superfusion, 5 min fractions of superfusate were collected using an LKB "Redirac" fraction collector, the tray of which had been packed with crushed ice, surrounding the sample tubes. After the experiment, these samples were assayed for lactate dehydrogenase activity (section 3.17).

The effluent from Porapak Q chromatography was used to estimate the potassium ion content of the tissue (section 3.16).

SUMMARY OF EXPERIMENT.

PREPARE STRIATAL SLICES (SECTION 3.1)

↓

INCUBATE FOR 0 TIME,
15 min OR 1 hour (SECTION 3.2) → COLLECT EFFLUENT FOR LDH ASSAY (SECTION 3.17).

↓

EXTRACT ENKEPHALINS.

CENTRIFUGE. → ASSAY PELLET FOR PROTEIN (SECTION 3.18)

PASS SUPERNATANT OVER PORAPAK Q (SECTION 3.13 c). → ASSAY WASH FOR K^+ (SECTION 3.19)

↓

SEPARATE AND PURIFY ME AND LE BY HPLC (SECTION 3.14)

↓

ASSAY ME AND LE USING ELISA (SECTION 3.17).

3.11 . EFFECT OF INHIBITORS OF ENKEPHALIN METABOLISM ON THE ENKEPHALIN CONTENT OF STRIATAL SLICES.

The compounds bestatin and SQ 24994, which had been shown to protect LE from enzymic degradation _in vitro_, were tested on corpus striatum slices _in vitro_, for possible effects on the endogenous ME and LE content of the slices. The striatal slices (section 3.1) were superfused for 1 h in the apparatus described in section 3.2, using a 1.5 ml chamber through which Krebs-Ringer solution flowed at 0.2 ml/min. The test compounds were dissolved in the supply of Krebs-Ringer solution. Four different conditions were examined:

NO DRUGS (CONTROL)	n = 4
SQ 24994 10 uM	n = 4
BESTATIN 100 uM	n = 4
SQ 24994 10 uM + BESTATIN 100 uM	n = 4

After 1 h superfusion, the slices were removed from the apparatus and drained. A tissue extract was made and ME and LE were purified and separated as described in sections 3.13 and 3.14. The tissue content of ME-like or LE-like immunoreactivity was measured by the ELISA method (section 3.15).

3.12 . *In Vivo* ADMINISTRATION OF SQ 24994.

(a) Drug solutions.

The compound was administered dissolved in an "artificial CSF"* of the following composition (mM); NaCl, 126.5; KCl, 3.0; $KH_2PO_4 \cdot 2H_2O$, 0.5; $NaHCO_3$, 26.2; $CaCl_2$, 1.8; $MgSO_4$, 0.41; glucose, 3.6; urea, 3.3. (Marks and Rodnight, 1972). The solution for injection contained the drug at 3.2 mM; the 20 µl injection volume therefore contained 64 nmol SQ 24,994, so that its concentration in the ventricular cerebro-spinal fluid would be expected to lie in the µM range. "Control" animals received saline alone.

(b) Treatment of animals.

Male Sprague-Dawley rats weighing 160 - 180 g were anaesthetized with halothane. The head was fixed in a stereotaxic frame, the skull surface exposed, and a burr hole drilled 0.2 mm caudally and 1.7 mm laterally, from the bregma. Injections were made using a 100 µl Hamilton syringe; the depth of penetration was limited by a rubber disc fixed 3.8 mm from the tip of the needle. These co-ordinates were chosen to ensure that the drug solution was injected into the lumen of the lateral ventricle. Injections were made over a period of about 1 min to avoid a sudden rise in CSF pressure. Five "control" rats received artificial CSF alone, and six were treated with SQ 24,994. The scalp was closed with suture clips.

Animals recovered from anaesthaesia within a few minutes. They were sacrificed 30 min after injection of the drug.

(c) Extraction of enkephalins.

An acid extract was made of the whole brain minus the cerebellum, and ME and LE purified from the extracts as described in sections 3.13 and 3.14. ME and LE were measured using the ELISA technique (section 3.15).

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* "CSF" is cerebro-spinal fluid.

3.13 . EXTRACTION AND PURIFICATION OF ENKEPHALINS.

(a) Homogenization.

Tissues were extracted into 5% aqeous acetic acid, which had been adjusted to pH 1.5 by the addition of HCl. This solution was cooled to approx. 4° C before use. A 10 ml, glass/PTFE homogenizer was used to disperse the tissue sample into 10 ml of the CH_3CO_2H/HCl solution; the homogenizer was kept cold in ice during use. The extract was transferred to a centrifuge tube standing in ice. A further 5 ml of extraction medium was used to wash the homogenizer and pestle and was then pooled with the extract.

(b) Centrifugation.

The homogenate was centrifuged at 45,000 x g for 45 min in a Sorvall centrifuge refrigerated to 4° C. The pellet was retained, to be assayed for protein content. The supernatant was immediately passed over Porapak Q.

(c) Porapak Q chromatography.

A 0.4 ml bed of this hydrophobic resin was packed in an LKB disposable "Econocolumn". The resin was cleaned by three cycles of alternate washings with 15 ml, 95% ethanol, then 15 ml distilled water; finally additional water (at least 25 ml) was passed through the column before the application of the sample. The supernatant from centrifugation was run through the gel, followed by 10 ml distilled water. The column was eluted with 8 ml methanol; the eluate was collected in a 50 ml, silanized pear-shaped flask. Eluates were rotary-evaporated to dryness at 30° C. The dried material was stored in a sealed flask at -20° C before HPLC purification.

3.14 . SEPARATION OF ME FROM LE BY HPLC.

Running conditions are described in section 3.5.

(a) Calibration.

1 μg each pure ME and LE, dissolved in 180 μl running solvent, was injected. Retention times were obtained by measuring the chart paper.

(b) Sample loading.

Samples were redissolved in 200 μl running solvent and 180 μl injected onto the column.

(c) Sample collection.

Fractions corresponding in elution time to ME and LE were collected by hand into silanized 25 or 50 ml pear-shaped flasks. After rotary evaporation sample flasks were stored at -20° C for ELISA.

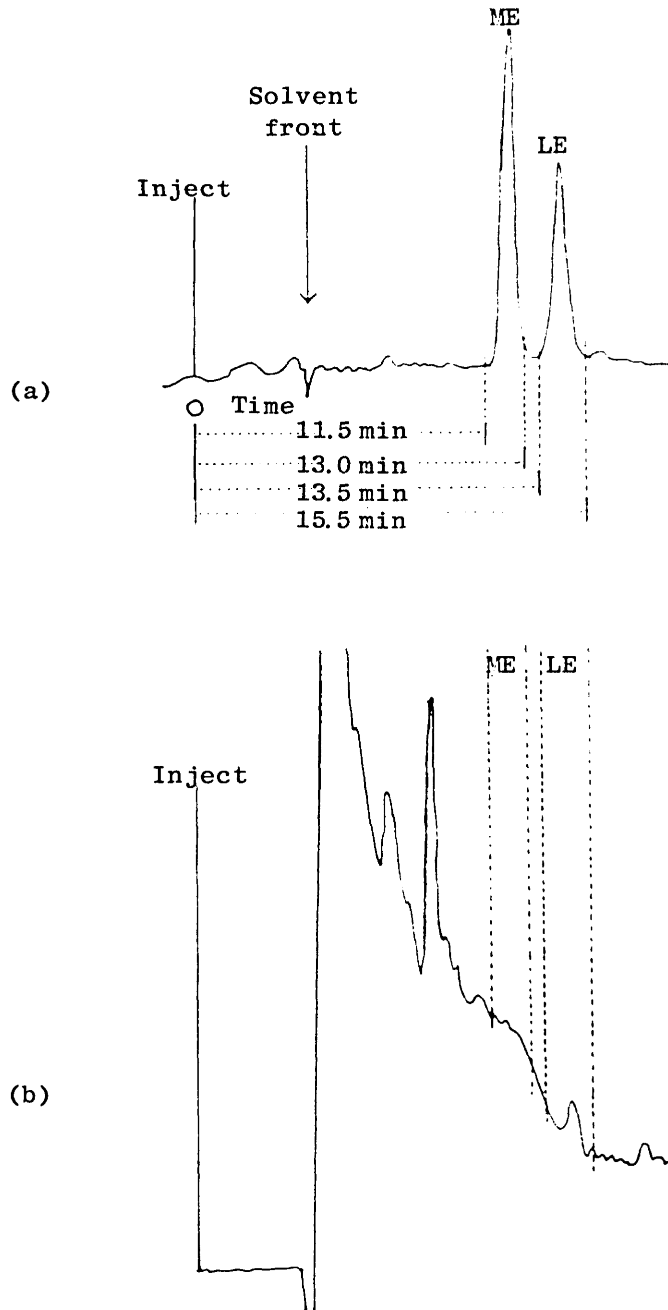


Fig. 3.14: Separation of LE from ME by HPLC. (a) 1 ug each pure LE and ME. (b) Tissue extract (Experiment 3.12). Running conditions and equipment are described in Sections 3.5 and 3.14 Fractions corresponding in elution time to authentic ME and LE standards were collected for quantification by the Elisa method (Section 3.15).

3.15. ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR ME AND LE.

(a) Principle of the assay.

Immunoreactive molecules in solution are detected and quantified by their ability to compete for binding to a specific antibody in solution with a specific antigen immobilized to a solid phase. The assay was based on that described by Zamboni et al (1983).

(b) Method

(i) Immobilized antigen is coated in the wells of a polystyrene micro-titre plate. ME or LE was covalently linked to ovalbumin using glutaraldehyde; the peptide-protein conjugate was purified by gel-filtration chromatography on a G-10 Sephadex column, 1.5 by 50 cm. The protein fractions were lyophilised and reconstituted in 0.05M bicarbonate buffer pH 9.6. The concentration of the ME-ovalbumin conjugate in the "coating buffer" was 1.5ng/ml; that of the LE conjugate was 5ng/ml. Wells containing 150 µl "coating buffer" were incubated at 4° C for 18 to 24 h. Each well was then washed three times with borate buffered saline ("buffer B": 100mM H_3BO_4, 26mM $Na_2B_4O_7$, 100mM NaCl 0.05% Tween -20, 1 mg/ml BSA, pH 8.4 at 4° C).

(ii) Reagents, dissolved in 50 µl buffer B, were added to the antigen-coated wells as follows:-

(I) standard dilutions of peptides (ME or LE) or unknown samples.

(II) specific rabbit-anti ME or -anti LE antibody. The rabbit-anti ME serum had been raised against a ME-snail haemocyanin conjugate and was used at a final dilution of 1:48,000; the anti LE serum had been raised against a conjugate of LE to bovine serum albumin and was used at a final dilution of 1:6,000.

(III) a second, anti-immunoglobulin antibody conjugated to a marker enzyme; in this case a 1:250 dilution of goat anti-rabbit IgG which had been covalently linked to the enzyme.

horseradish peroxidase. Plates were incubated at 4° C for 3 h for LE, or for 6 to 14 h for ME.

(iii) The wells were then washed 4 times with buffer B, removing soluble antigen-antibody complexes. The wells now contained only that specific antibody which had bound to the immobilized antigen.

(iv) The specific antibody bound to the plate was then quantified by measuring the activity of the horseradish peroxidase, which was linked to it via the second antibody. 150 µl of o-phenylene diamine substrate in a citrate/phosphate buffer (100 mg substrate and 40 µl of 30% hydrogen peroxide per 100 ml buffer, which contained 24 mM citric acid and 51 mM di-sodium hydrogen phosphate, pH 5.0). Reaction proceeded at room temperature. The amount of enzyme present on the plate, and hence the intensity of colour, will depend on the amount of specific antibody bound to the plate, and this in turn will be reduced according to the amount of soluble antigen which was originally present. 30 to 60 min. after the addition of substrate, the reaction was stopped by adding 50 µl, 3.75 M sulphuric acid to each well. The absorbance of the chromogen was read at 492 nm in a "Titertek Multiskan" automated ELISA reader.

Each plate of 8 by 12 wells contained standards of synthetic ME or LE (absorbances Ax) enzyme blanks (no specific serum, absorbance A_B) and wells without free antigen (Ao). It had been shown previously (Zamboni et al, 1983) that the enzyme blank had the same absorbance as wells containing non-immunized rabbit serum or wells without ovalbumin conjugated peptides.

At each standard ME or LE concentration, the A_B value was subtracted from Ax and the "corrected" Ax divided by the similarly corrected Ao. The corrected Ax/Ao ratio was plotted against the logarithm of the standard soluble peptide concentration (figure 3.16 a).

(c) The interface of purification and assay.

It had been found in preliminary experiments that biological samples which had been purified by HPLC (section 3.15) appeared to contain approximately one-and-a-half times more ME or LE when measured by ELISA, than when they were bio-assayed on the mouse vas deferens preparation (Drs. PM Caesar and G Zamboni, unpublished data). This was attributed to the introduction during HPLC of some material which reduced the amount of colour developed during ELISA, by interfering either with the antigen-antibody reaction or with the enzyme-chromogen reaction (figure 3.16 b). The latter possibility was discounted by the demonstration that HPLC column effluent had no effect on the enzyme/substrate reaction (data not presented). The tri-fluoroacetic acid in the HPLC solvent was considered the most likely cause of the problem.

The inclusion of 5% new-born bovine serum in the assay buffer eliminated the interference with the LE assay but was of no advantage in the ME assay. Before use in the ELISA, ME standards were therefore routinely passed through the HPLC system, in exactly the same way as biological samples.

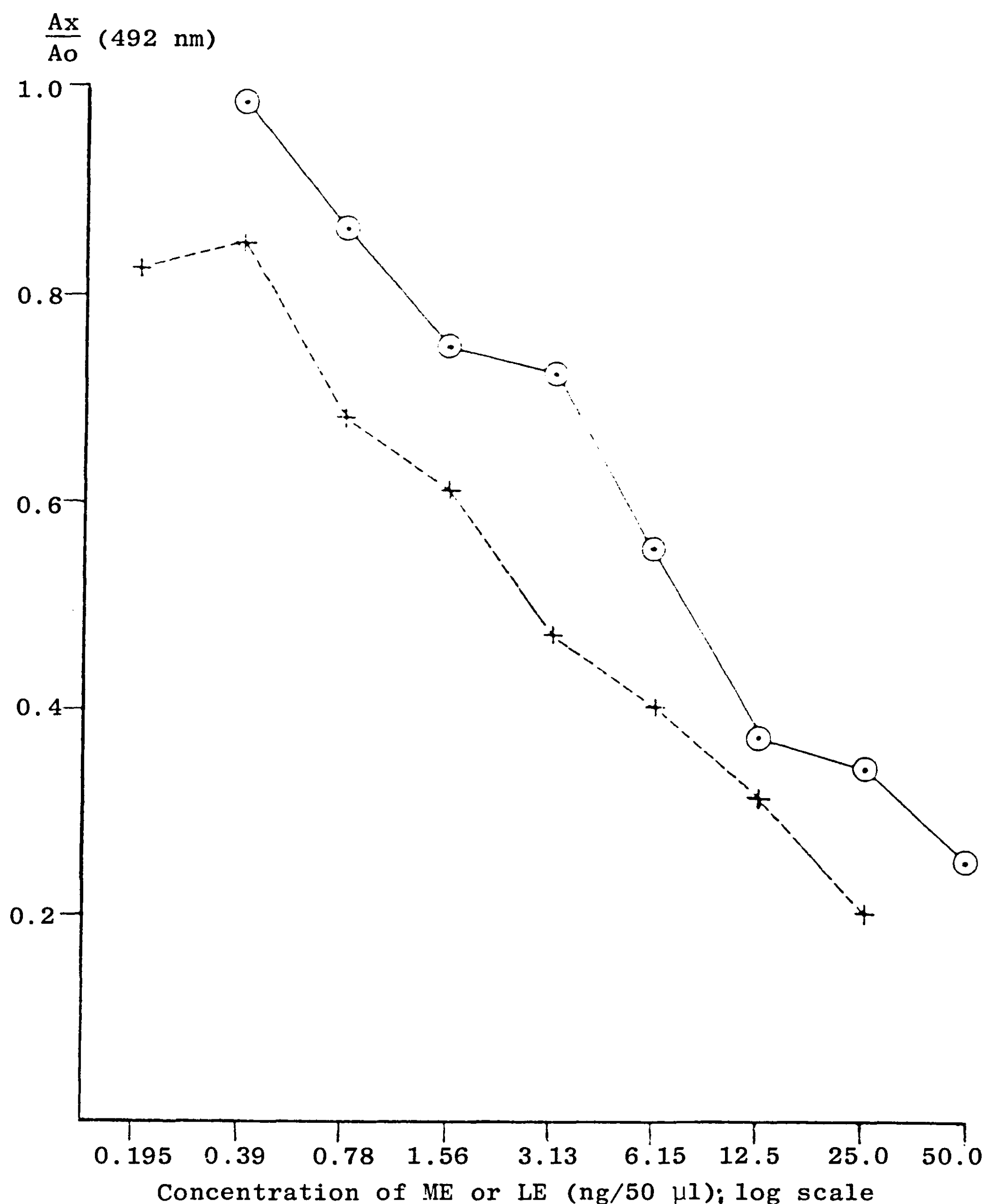


Fig. 3.15(a): Calibration curve for the enzyme-linked immunosorbent assay (Elisa). Absorbance A at 492 nm is a measure of the extent of o-phenylene diamine oxidation by the peroxide bound to anti-immunoglobulin second antibody.

$Ax = A'x - A$; $Ao = A'o - A$.

A'x is absorbance of a well in which a known quantity of soluble antigen (ME or LE) had competed with immobilised antigen for binding to the specific anti-ME or anti-LE serum.

A'o is the absorbance of "control" wells (no soluble antigen); A is the absorbance of "blank" wells.

⊙ ME (using "ME-OE" serum); + LE (using "L3" serum)

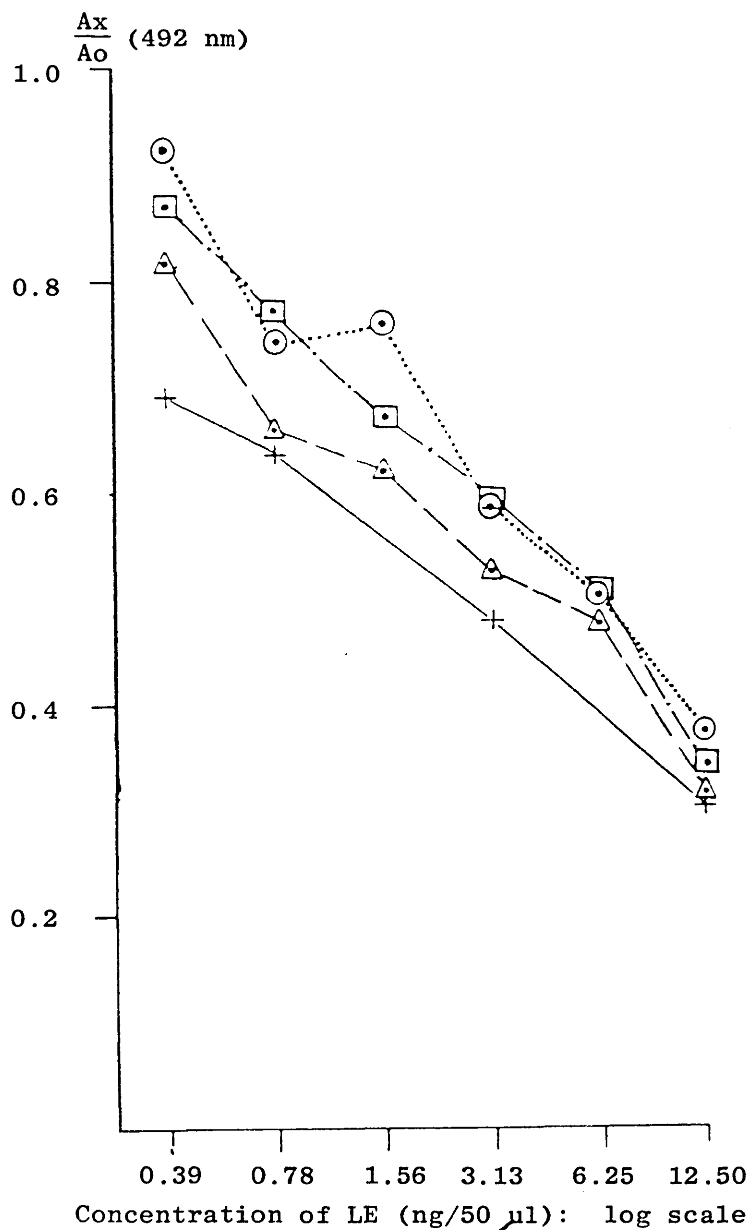


Fig. 3.15(b): The effects of HPLC purification of leucine enkephalin on its immunoassay by the ELISA method.

⊡ Standard curve obtained with LE which had not been chromatographically treated.

+ Standard curve for LE which had been passed through HPLC (Section 3.14).

△ Standard curve obtained when LE standards were dried-down *in vacuo* and reconstituted in 0.02% trifluoracetic acid.

⊙ Standard curve obtained when LE standards, which had been subjected to HPLC, were assayed in the presence of 5% foetal calf serum.

3.16. MEASUREMENT OF TISSUE POTASSIUM CONTENT.

(a) Apparatus.

A Gallenkamp "Na-K flame analyser" was used for quantitative flame emission photometry.

(b) Calibration.

The standards consisted of potassium chloride (AR) solutions in distilled water, at concentrations ranging from 100 to 500 μM in 20 μM increments. A graph of mean scale deflection (n = 2) versus K^+ concentration was found to be linear over the range tested.

(c) Sample preparation.

The samples originated from an experiment in which the tissue enkephalin content of corpus striatum slices was measured, after they had been superfused _in vitro_ for different lengths of time. Tissue was homogenized in acetic acid/HCl and the extract centrifuged as described in section 3.1 The 15 ml extract was applied to the Porapak Q column and the effluent was collected: inorganic ions do not bind to the column. Three, 1 ml portions of distilled water were run down the column and these washings combined with the sample effluent. The final 18 ml samples were stored frozen at -20° C until required for K^+ assay.

3.17 . MEASUREMENT OF LACTATE DEHYDROGENASE ACTIVITY.

(a) Apparatus.

Ultraviolet absorption was monitored using a Cary model 210 recording spectrophotometer.

(b) Materials.

Solution I ; 50 mM potassium phosphate buffer pH 7.5 containing 3 mg sodium pyruvate per 80 ml.

Solution II ; 10 mg Na_2NADH in 1.5 ml solution I.

(c) Method.

2.85 ml solution I and 0.05 ml solution II were mixed in a 3 ml quartz u.v. cuvette. 0.1 ml sample (Krebs solution) was added and the decrease in absorption at 340 nm was followed relative to a reference cuvette containing 0.1 ml pure Krebs-Ringer solution in place of sample. The presence of a high concentration of pyruvate or other reducible substrate in some of the samples might lead to spuriously high rates of NADH oxidation. Several of the samples were checked by omitting pyruvate from the stock solutions; the rate of fall in O.D. was negligible. The changes in NADH concentration were calculated using the Beer-Lambert law assuming an extinction coefficient of 6.22 cm^2 per μmol.

3.18. PROTEIN ASSAY.

Samples of tissue were dispersed in 2M NaOH, if necessary by means of a "sonicator".

Before assay the tissue suspensions were incubated at room temperature for about 24 h, and mixed at intervals to assist solubilization. The assay technique was based on that of Lowry _et al_ (1951) except that an automated sample analyzer was used. The assay was calibrated against standards of bovine serum albumin at concentrations from 20 to 100 µg/ml; the standard curve was approximately linear over this range. Each sample was tested at 3 or 4 different dilutions.

3.19. EFFECTS OF THE COMPOUND SQ 24994 ON THE MOUSE VAS DEFERENS BIOASSAY.

(a) Equipment.

Grass Instruments dual channel physiological stimulator.

Churchill thermostatic pump.

Lectromed "Devices" pre-amplifier and chart recorder.

Isometric transducers.

1 ml, upward-flow organ baths.

(b) Method.

The vas deferens preparation was set up according to Hughes *et al.* (1975). The Krebs-Ringer solution was of the following composition (mM): NaCal, 133.3; KCl, 4.7; $CaCl_2$, 2.5; NaH_2PO_4, 1.38; $NaHCO_3$, 1.63; glucose, 11.1. This medium was gassed both in the reservoirs and in the organ bath with a 95% O_2, 5% CO_2 mixture. The vas deferens was suspended between two platinum ring electrodes which were used for field stimulation, the wave-form of which was as follows:-

Train rate	0.06 Hz
Train duration	200 msec
Pulse frequency	40 Hz
Pulse width	200 usec
Pulse output	20-30 V

These electrical pulses cause the tissue to contract with a uniform amplitude; opioids inhibit this twitch response in a dose-dependent manner. Dose-response curves were constructed by adding doses of from 1 to 40 ng LE, producing concentrations in the organ bath from 1.8 to 72 nM; the preparation was washed between doses.

A single dose of 2 µg SQ 24994 (6.67 µM) was added to the organ bath, and pre-incubated with the tissue for 15 min; a second series of LE doses was then given. To maintain a uniform concentration of SQ 24994 a further 2 µg of the drug was added to the bath before every LE dose.

R E S U L T S

4.1 DE-SALTING SAMPLES FROM EXPERIMENTS ON THE BREAKDOWN OF ^3H LE .

It has been described in section 3.7 how the procedure finally selected used the strong cation-exchange resin , Dowex 50-W . To assess the recovery of labelled material a standard mixture containing 30 pmol each of tritium-labelled LE and Tyr , was made up in the Ringer solution used for the incubations , to mimic the conditions of sample application (table 4.1 a). As shown by the data of table 4.1(b) a majority of the sample eluted with the first 1 ml of 10% NHs eluent - the 5 ml used in practice therefore eluted a maximum amount of sample . Table 4.1(c) demonstrates that little sample material was lost during preliminary washings of the column , which eluted the inorganic salts .

4.1. DESALTING BY DOWEX COLUMN CHROMATOGRAPHY.

TABLE 4.1 (a). RECOVERY OF 30 pmol LE AND 30 pmol Tyr.

STANDARD	% RECOVERY ± S.E.M. (n = 4)
LE	80.0 4.3
Tyr	77.0 6.9

TABLE 4.1 (b). EFFECT OF ELUTION VOLUME ON THE RECOVERY OF 30 pmol LE

VOLUME OF 10% NH_3 (ml)	% COUNTS RECOVERED MEAN ± S.E.M. (n = 4)
0 - 1	62 3
1 - 2	29 4
2 - 5	10 3

TABLE 4.1 (c). ELUTION OF 30 pmol LE AND 30 pmol Tyr; % MATERIAL IN EACH FRACTION.

STANDARD	8 ml HCl	25 ml H_2O	4 ml NH_3 (10%)
LE	2.4	0.3	73
Tyr	5.8	1.9	80

COLUMN CHROMATOGRAPHY PROCEDURE IS DESCRIBED IN SECTION 3.9 c.

4.2. BEHAVIOUR OF LE AND ITS CATABOLITES ON HPLC .

High performance liquid chromatography is potentially a rapid and convenient method of separating mixtures of peptides , and high recovery is possible . The HPLC system , using a reverse phase analytical column , was tested for this purpose as described in section 3.5 .

Table 4.2 records the elution times of the components of a mixture containing 3 µg of each of LE and its four breakdown-products ; the resolution was found to be unsatisfactory .

TABLE 4.2. SEPARATION OF LE CATABOLITES BY HPLC (SECTION 3.)

PEAK	ELUTION TIME/min
SOLVENT FRONT	4.50
Y	10.00
YG	10.50
YGG	10.00
YGGF	13.50
YGGFL	17.25

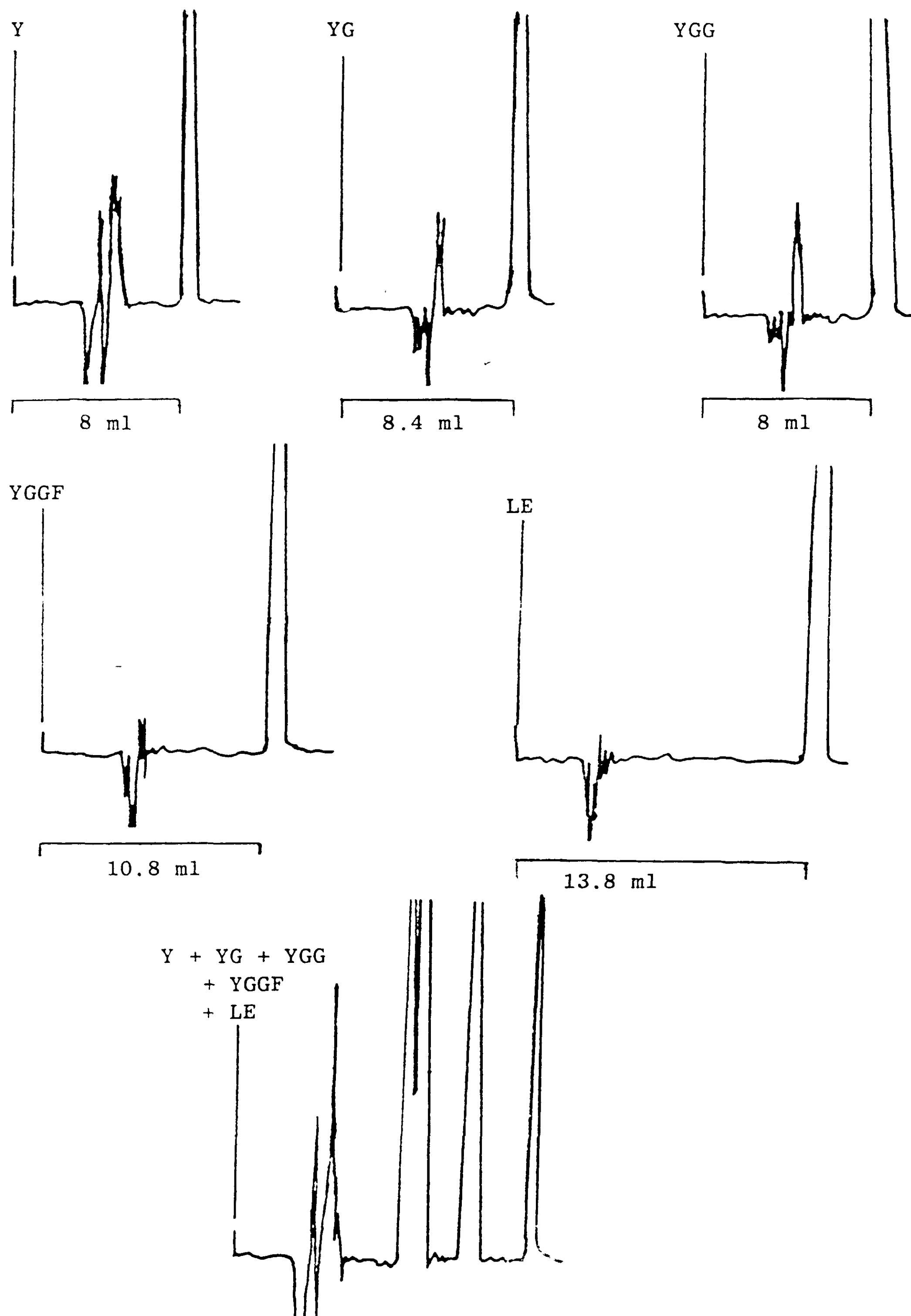


Fig. 4.2: Separation of LE catabolites by HPLC. Attempted separation of 4 labelled (i.e. tyrosine-containing) catabolites of $^{3}H-(Tyr^{1})$-LE by high performance liquid chromatography on a "Techsphere C-18" column under conditions described in section 3.5 . 3 µg of each peptide standard, dissolved in 50 µl of the eluent, was run at a flow-rate of 8 ml/min. Peptides were detected by UV absorbance at 210 nm. Single-letter code for amino-acids:- Y = Tyr; YG = Tyr-Gly; YGG = Tyr-Gly-Gly; YGGF = Tyr-Gly-Gly-Phe.

4.3.1. SEPARATION OF LE AND ITS BREAKDOWN PRODUCTS BY THIN-LAYER CHROMATOGRAPHY.

Section 3.6 (a) outlines the testing of a range of solvent mixtures, in an attempt to separate the five components on silica TLC plates. The system ultimately selected (mixture "f") was used as described in section 3.6(b) to resolve a mixture containing 1 ug each of LE and its four breakdown products.

To provide the data presented in tables 4.4.1.1.(a) to (e) and table 4.4.2, the zones on the TLC plate occupied by the radio-labelled products were visualised by adding unlabelled "carrier" standards to every sample before chromatography. The Rf's of components of experimental samples after de-salting were close to those of markers chromatographed from methanol.

Table 4.3. Separation of peptide standards.

STANDARD	Rf spotted individually in 50 µl Methanol	Rf spotted as a mixture in 50 µl Methanol	Rf spotted as a mixture in 50 µl Krebs medium
YGGFL	0.76	0.76	0.70
YGGF		0.65	
YGG	0.32	0.32	0.28
YG	0.44	0.44	0.38
Y	0.58	0.58	0.50

Solvent front migrated 15 cm.

Band width 0.5 - 1 cm.

4.3.2. RECOVERY OF PEPTIDES FROM THIN LAYER CHROMATOGRAPHY.

The data from the experiments on the breakdown of ^{3}H LE can only be interpreted provided that, within any sample, LE and its degradation products are all represented by scintillation counts in a constant proportion to their concentrations in the Krebs medium, at the end of the incubation period. If this assumption is correct then, since the total radioactive concentration was the same in all samples at the start of incubation, the total radioactivity recovered as breakdown products should be the same, irrespective of the peptide composition of the sample. The total recovered dpm was therefore calculated under conditions where there were wide differences in the peptide composition of the sample (Table 4.3.2.a).

These totals were examined for differences using a parametric analysis of variance (F test). Exhaustive partitioning of the sums-of-squares revealed only very low values of F and no differences approaching significance, i.e., the mean recovery does not differ significantly between treatments.

The experiment was conducted using nine different preparations of striatal membrane; each preparation received numerous "treatments" (different compounds at different concentrations; incubations without striatal membranes were also set up).

The single factor analysis of variance ("anovar") assumes a fully-randomized population. It may be more realistic to analyse the data for significant mean differences in recovery by comparing the recoveries under different treatments within each experiment. The "no inhibitor" and "no tissue" incubations are the two treatments with the largest number of replicates. The results from these treatments were examined in two ways :-

(i) As though the two treatments were independent populations (as in the anovar above) by means of (a) Student's "t" test on the difference between mean total dpm of the two treatments; (b) Mann

TABLE 4.3.2. (a)

TOTAL RADIOACTIVITY RECOVERED FROM TLC : EFFECT OF DRUG TREATMENTS.

TREATMENT	n	TOTAL DPM RECOVERED	% RECOVERY
NO TISSUE	9	23887 ± 5988	8.9 ± 2.2
CONTROL - NO INHIBITORS	9	35016 ± 9625	13.0 ± 3.4
SQ 24,994 10 μM	5	30013 ± 14514	11.2 ± 5.4
THIORPHAN 10 μM	5	26423 ± 23533	9.8 ± 8.8
BESTATIN 100 uM + SQ 24994 10 μM	5	23838 ± 20161	8.9 ± 7.5
BESTATIN 100 uM + THIORPHAN 10 μM	4	28769 ± 27149	10.7 ± 10.1
PUROMYCIN 100 μM	6	29468 ± 20540	10.9 ± 7.6
POOLED DATA	43	28451 ± 24489	10.4 ± 8.9

DATA PRESENTED AS $\bar{x} \pm$ S.E.M.

DATA WAS SUBJECTED TO ANALYSIS OF VARIANCE.

BETWEEN ALL TREATMENTS $F = 0.2$; $p > 10\%$

EXHAUSTIVE PARTITIONING OF THE SUMS-OF-SQUARES REVEALED ONLY VERY LOW VALUES OF F AND NO DIFFERENCES APPROACHING SIGNIFICANCE.

and Whitney's "U" test, comparing the difference between treatment medians.

(ii) As though each pair, "no tissue" and "no inhibitor", prepared from a common pool of striatal membranes, constituted one experiment; the difference in total recovered dpm was examined by (a) the paired sample"t" test; (b) Wilcoxon's signed rank test (T statistic).

The results in table 4.3.2. b show that there is a significant mean difference in recoveries between "no tissue" and "no inhibitor"; the only difference between the two treatments is the presence of the striatal membranes in the latter. This result also supports the paired sample approach as a more realistic way of analysing the data. The standard statistical test of data structured in this way, where there are many different treatments, is the "randomized block".

Unfortunately this approach requires that the model be incorporated into the original experimental design, and that all treatments have equal numbers of replicates.

In order to determine whether this difference in recovery is common to all incubations containing the membrane preparation, all such incubations listed in table 4.3.2(a) within each experiment were compared with the "no tissue" incubations in their respective experiments. To do this, Wilcoxon's test was used as follows. The dpm values of each "no tissue" incubation are subtracted from each in turn of the incubations containing membranes, within the same experiment. These differences within each experiment were then ranked and the median value selected. Applying this procedure to all 9 experiments gives 9 median differences from which the Wilcoxon T statistic was computed in the usual way. The median differences were significant at $p = 0.05$. Although this is not the conventional form of Wilcoxon's test it is reasonable to conclude that there is a genuine difference in overall recovery between "no

TABLE 4.3.2. (b)

COMPARISON OF TWO DIFFERENT ASSUMPTIONS ABOUT THE STRUCTURE OF THE DATA FROM THE BREAKDOWN EXPERIMENTS.

ASSUMPTION A : that all the data has come from a randomized population - there are no particular links between values for different treatments arising from one experiment.

ASSUMPTION B : that values for the two treatments arising from each experiment are "paired", differing only in the presence or absence of the striatal membranes. (Any one experiment of the 9 used a single batch of reagents and of membranes; new materials were prepared for every experiment).

		NO TISSUE	NO INHIBITOR
A	MEAN $\pm$ 95% CONFIDENCE LIMITS	23887 $\pm$ 13772	35016 $\pm$ 22138
	DIFFERENCE BETWEEN MEANS $\pm$ 95% CONFIDENCE LIMITS	11,129 $\pm$ 2403	
	t ; p	t = 0.98	$p > 5\%$
	MEDIAN $\pm$ 95% CONFIDENCE RANGE	22707; 14516/78094	14399; 10362/52006
	DIFFERENCE BETWEEN MEDIANS	8308	
	U ; p	U = 28	$p > 5\%$
B	MEAN DIFFERENCE $\pm$ 95% CONFIDENCE LIMITS	11,128 $\pm$ 9892	
	t ; p	t = 2.59	$p < 5\%$
	MEDIAN DIFFERENCE $\pm$ 95% CONFIDENCE RANGE	4726 ; -422/+22742	
	T ; p	T = 4	$p < 5\%$

tissue" samples and those from all other treatments. This test was applied reiteratively to compare each treatment in turn with all the others. No significant differences were found. Each treatment was also compared with each of the others in turn, by applying the Wilcoxon procedure in the normal way; the paired sample t test was also used. No other difference in recovery approached significance. The conclusion was drawn that only the presence or absence of striatal membranes affected recovery.

It was found that within - treatment variability could be reduced by expressing the dpm for a treatment as a percentage of the "no tissue" dpm within the same experiment (table 4.3.2. c). By thus reducing the effects of between - experiment variations, the differences between treatments emerge more clearly. The results of the analysis of variance show that there are no significant differences in recovery between treatments, although the peptide compositions varied considerably. This justifies the assumption that the recoveries of LE and of its four labelled degradation products are all equal.

TABLE 4.3.2.c "TREATMENT" RESULTS WITHIN EACH EXPERIMENT, EXPRESSED AS % OF THE "NO-TISSUE" RESULT WITHIN THAT EXPERIMENT.

TREATMENT	n	TREATMENT DPM ÷ NO TISSUE DPM (%)
CONTROL-NO INHIBITORS	9	145.7 ± 14.6
SQ 24,994 10 µM	5	139.8 ± 20.9
THIORPHAN 10 µM	5	111.9 ± 16.5
BESTATIN 100 µM + SQ 24994 10 µM	5	112.3 ± 13.7
BESTATIN 100 µM + THIORPHAN 10 µM	4	124.1 ± 18.8
PUROMYCIN 100 µM	6	126.0 ± 12.5

RESULTS GIVEN AS $\bar{x} \pm$ S.E.M. %

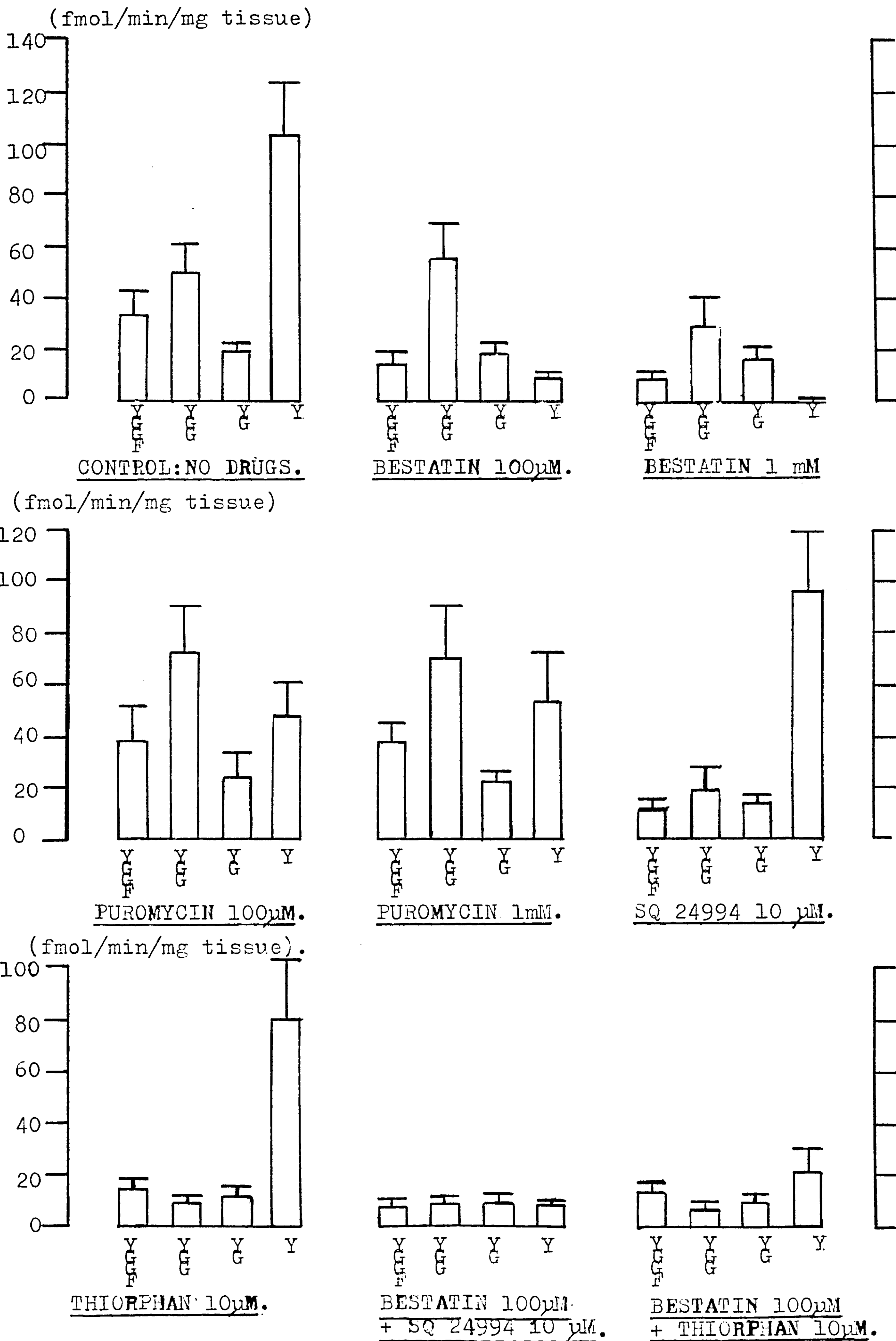
ANALYSIS OF VARIANCE GAVE "BETWEEN TREATMENTS" $F = 0.79$
$p > 10\%$

ALL POSSIBLE COMPARISONS BETWEEN TREATMENTS WERE MADE USING THE F TEST, BUT NO SIGNIFICANT DIFFERENCES WERE FOUND.

4.4.1. BREAKDOWN OF LE BY STRIATAL MEMBRANES.

^{3}H LE at a concentration of 8 nM was incubated for 15 min with a preparation of rat corpus striatum membranes (section 3.4) and the labelled metabolites separated (sections 3.6 and 3.7) and quantified (section 3.8). The results were calculated as described in section 3.9.
The data has been presented as a series of histograms (figure 4.4.1.1.a) and also as tables giving rates of product formation averaged over 15 min. (Tables 4.4.1.1.a-e). Where drugs exerted a dose-dependent effect either on LE breakdown or on the formation of a particular product, log-dose vs. response curves were plotted (figures 4.4.1.1.b to f).
In tables 4.4.1.1.a to 4.4.1.1.e, the rate of product formation in the presence of a test compound was in every case compared statistically with the rate of product formation in the absence of any compound (control). The rates of product formation in the presence of different compounds were also compared directly; the results have been summarised in tables 4.4.1.2.a to 4.4.1.2.g .
The histograms in figure 4.4.1.1.a give a general impression of the effects of the different treatments. In the control incubation (no inhibitors), the major product was tyrosine, followed by Tyr-Gly-Gly, then Tyr-Gly-Gly-Phe, then Tyr-Gly.
The types of enzymic cleavage of LE which could have formed these products have been described in section 1.1.
All the compounds have altered the pattern of product formation, and the relative amount of each product varies between compounds, suggesting that the different compounds selectively inhibit distinct pathways of LE breakdown. For example, SQ24994 rduces the amounts of the three peptide products but does not affect Tyr formation; there is probably a direct route of formation of each product from LE, rather than a system of sequential LE cleavage from its carboxy-terminus. Bestatin and puromycin seem to have different effects, and the effect of either is distinguishable from that of SQ24994 or thiorphan; the latter two substances appear to have similar effects.
The combinations of bestatin and either SQ24994 or thiorphan appear to have depressed the total quantity of products formed more than any of the compounds did when used alone.

Figure 4.4.1.1.(a). Products of Leucine Enkephalin degradation by a striatal membrane preparation.



This is also apparent when the effects of the four compounds are examined across a range of concentrations (figures 4.4.1.1.b to 4.4.1.1.e).
The breakdown of LE was decreased by either bestatin or puromycin ; this effect was concentration-depend nt. Bestatin had a greater effect (figure 4.4.1.1.b). When a range of concentrations of SQ24994 or thiorphan was tested in the presence of 100 µM bestatin the breakdown of LE was further suppressed, the degree of inhibition increasing with the concentration of SQ24994 or thiorphan.
In figure 4.4.1.1.c the three single points on the right hand side of the graph represent the effects of bestatin, SQ24994 or thiorphan alone. More LE appears to have been broken down in the presence of 1 µM bestatin than in the presence of 100 µM bestatin plus either 10 µM thiorphan or 10 µM SQ24994, although the total inhibitor concentration was much less.
The difference is not simply one of potency, because the effect of 10 µM SQ24994 or thiorphan appears smaller than that of bestatin at 10 µM (figure 4.4.1.1.b); this reinforces the impression that the mechanisms of LE protection by these compounds are different. In fact the substance had specific effects on the formation of different products.
The most obvious effect of bestatin or puromycin was a reduction in the quantity of Tyrosine, implying that an LE-hydrolysing aminopeptidase was inhibited (figure 4.4.1.1.d). This effect could account for a major part of the protection from degradation afforded to LE by either compound (tables 4.4.1.1.a and b) - but of the two inhibitors bestatin was more effective than puromycin at high concentrations, although puromycin seemed more potent at low concentrations, strongly suggesting that different enzymes were inhibited.
Bestatin also clearly affected the Tyr-Gly-Gly-Phe content of the incubations (figure 4.4.1.1.e) whereas puromycin had no such effect. This product could only have been formed from LE by direct carboxypeptidase activity (section 1.1), which appeared also to be inhibited by SQ24994 or thiorphan. Thus these compounds have different, yet overlapping selectivities. Tyr-Gly-Gly might have been formed by sequential carboxypeptidase activity, or it might have been cleaved directly from the pentapeptide, by an endopeptidase or a dipeptidyl carboxypeptidase.

In the presence of 100 μM bestatin, the amount of Tyr-Gly-Gly was little different from control (figure 4.4.1.1.f), but when used either alone or in combination with 100 μM bestatin, either 10 μM SQ24994 or 10 μM thiorphan markedly reduced the Tyr-Gly-Gly concentration at the end of the incubation.

Tables 4.4.1.1.a to e contain the data from which these graphs were plotted and also present a complete picture of the quantitative effects of the inhibitors on each product, allowing conclusions to be drawn about the pathways of product formation and the enzymes involved.
Where a compound had a statistically significant effect on a product, this has been indicated in the tables.
The treatments were also compared with one another as part of the same statistical analysis (section 3.9) and these results have been gathered into tables 4.4.1.2.a to g .
The interpretation of the differences between treatments is pursued in section 5.2.2.

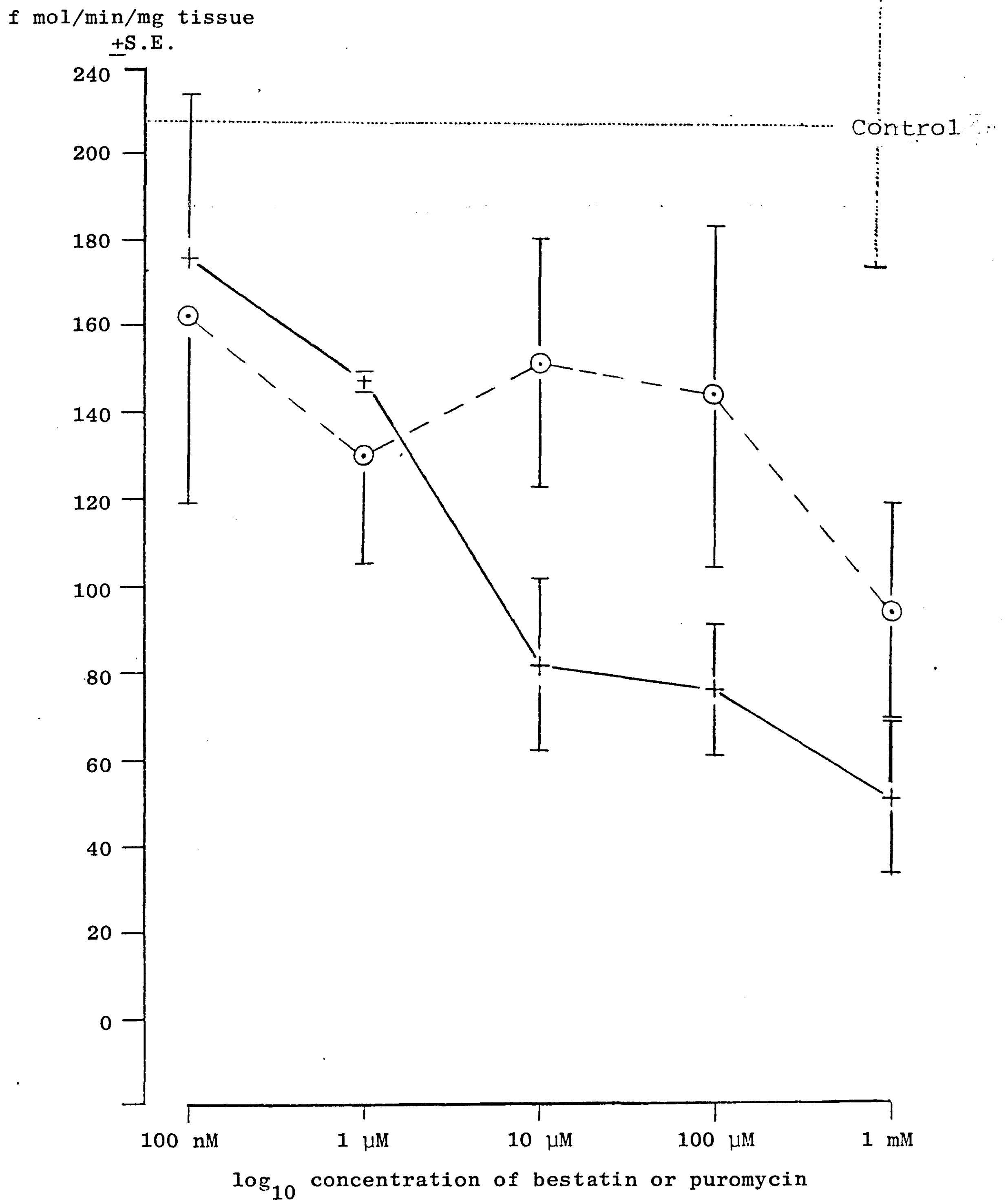


Fig. 4.4.1.1(b): Effects of bestatin or puromycin on the degradation of LE by a preparation of striatal membranes.

⊙ Puromycin

+ Bestatin

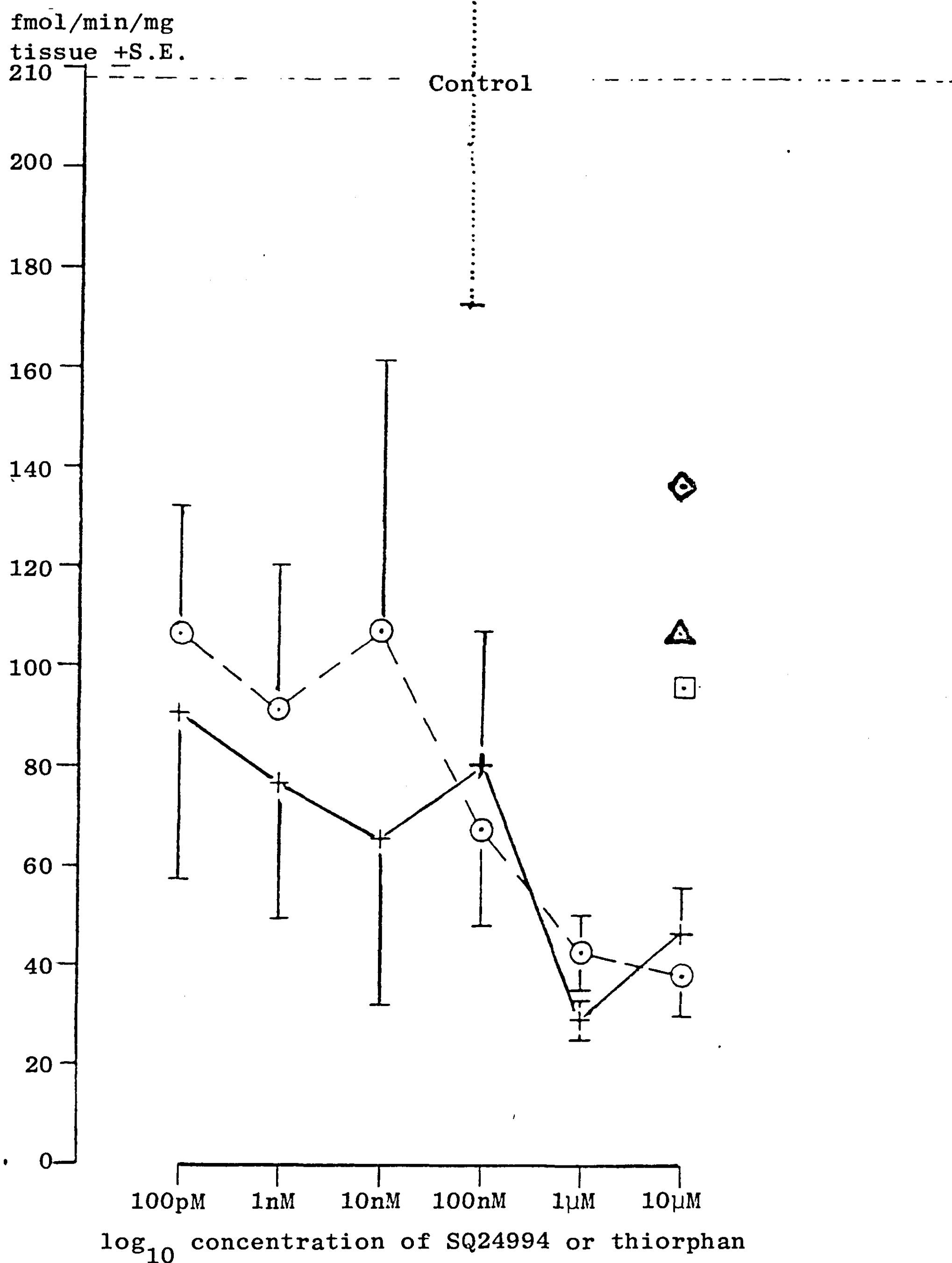


Fig. 4.4.1.1(c): Effects of a combination of 100 µM bestatin with either SQ24994 or thiorphan on the degradation of LE by a preparation of striatal membranes.

⊙ Effects of SQ24994 + 100 µM bestatin.

+ Effects of thiorphan + 100 µM bestatin.

◇ 10 µM SQ24994 only.

△ 10 µM thiorphan only.

⊡ 100 µM bestatin only.

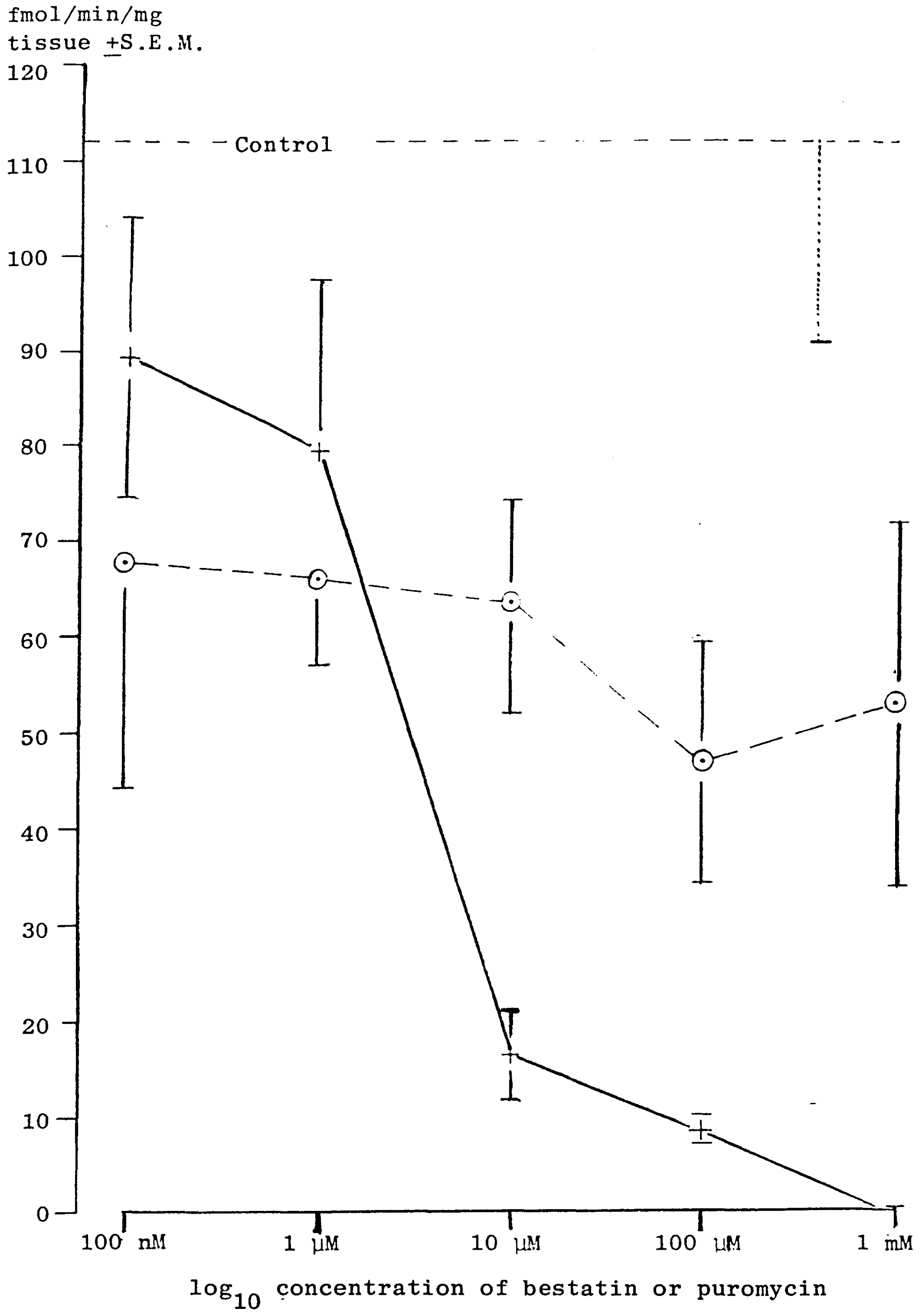


Fig. 4.4.1.1(d): Effects of puromycin or bestatin on the formation of tyrosine from leucine enkephalin by a preparation of striatal membranes.

⊙ Effects of puromycin

+ Effects of bestatin.

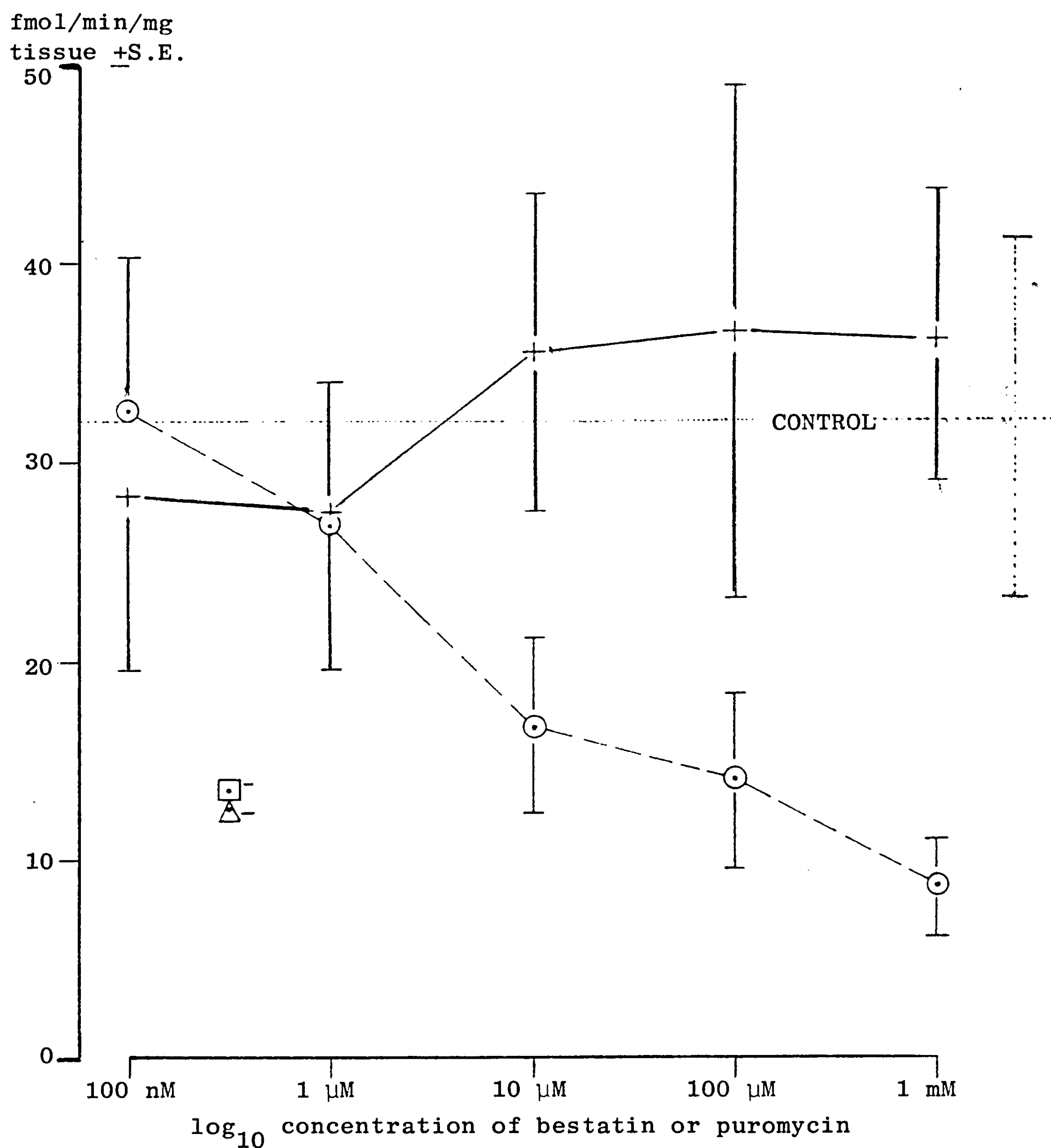


Fig. 4.4.1.1(e): Effects of bestatin or puromycin on the formation of Tyr-Gly-Gly-Phe from leucine enkephalin by a preparation of striatal membranes.

⊙ Effects of puromycin.

+ Effects of bestatin.

⊡ 10 μM thiorphan

△ 10 μM SQ 24994

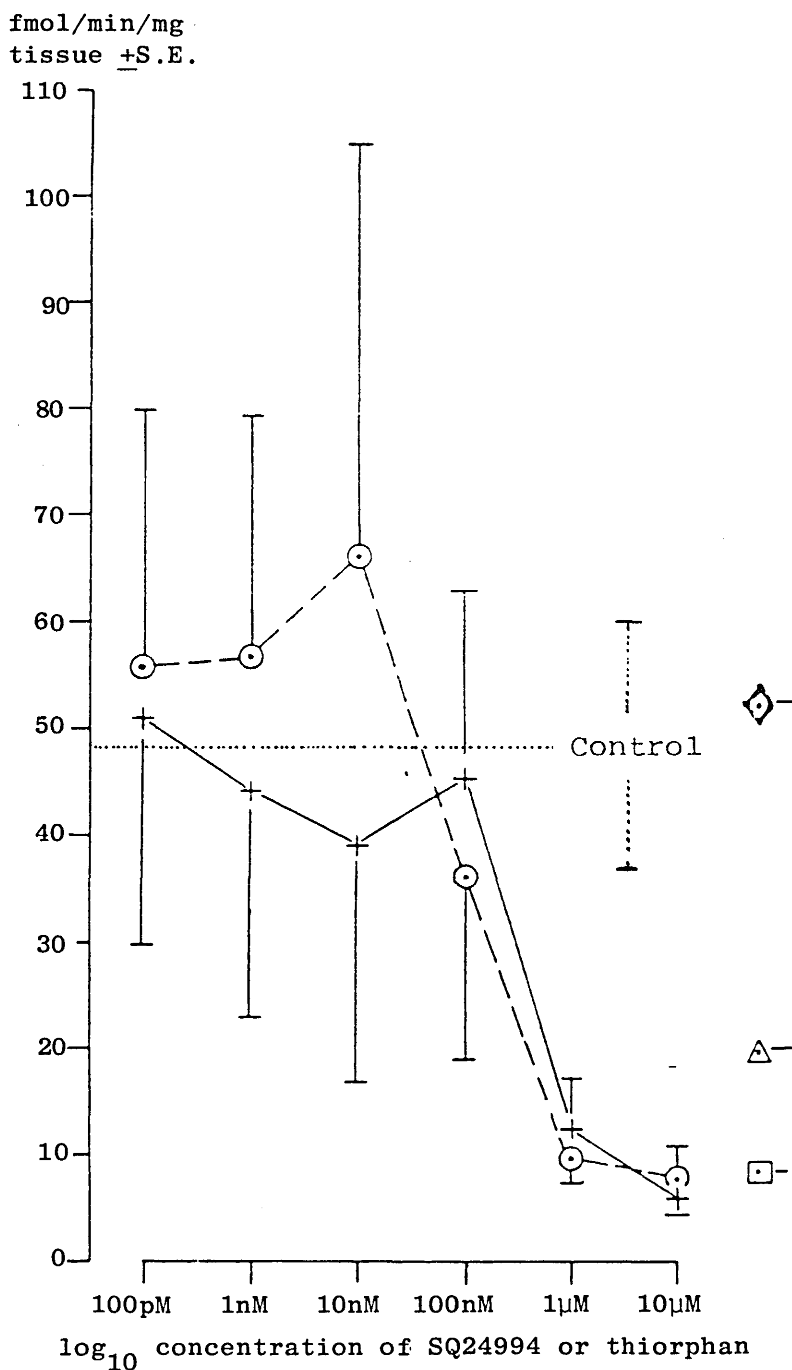


Fig. 4.4.1.1(f): Effects of a combination of 100 μM bestatin with either SQ24994 or thiorphan on the formation of tyr-gly-gly from leucine enkephalin by a preparation of striatal membranes.

+ Effects of thiorphan + 100 μM bestatin.

⊙ Effects of SQ24994 + 100 μM bestatin.

◇ 100 μM bestatin only.

△ 10 μM SQ24994 only.

□ 10 μM thiorphan only.

Table 4.4.1.1. (a). Breakdown of ^{3}H LE by striatal membranes: effects of bestatin.

Concentration	n	LE degraded	YGGF formed	YGG formed	YG formed	Y formed
Control	9	208.8 ± 35.3	32.2 ± 8.7	48.2 ± 12.2	18.0 ± 2.8	112.2 ± 21.0
100 nM	9	195.7 ± 38.1	32.7 ± 7.7	62.5 ± 18.2	23.9 ± 6.1	89.3 ± 14.7
1 μM	6	167.3 ± 2.5	26.9 ± 8.2	69.5 ± 15.7	24.7 ± 8.0	79.2 ± 18.3*
10 μM	8	101.6 ± 19.8**	16.7 ± 4.4	54.9 ± 14.4	14.8 ± 2.1	16.4 ± 4.5***
100 μM	8	96.0 ± 15.0**	13.9 ± 4.4*	54.6 ± 13.3	17.0 ± 4.5	8.6 ± 1.4***
1 mM	5	70.7 ± 16.8***	8.6 ± 2.3**	29.4 ± 10.8	16.5 ± 4.9	0.0 ± 1.1***

Values given are $\bar{x} \pm$ S.E.M.; units fmol/mg/min.

Statistical comparisons were made with control (no inhibitor) using analysis of variance.

Significance levels: * $0.05 > p > 0.01$

** $0.01 > p > 0.001$

*** $0.001 > p$

Table 4.4.1.1.(b). Breakdown of ^{3}H LE by striatal membranes: effects of puromycin.

Concentration	n	LE degraded	YGGF formed	YGG formed	YG formed	Y formed
Control	9	208.8 ±35.3	32.2 ± 8.7	48.2 ±12.2	18.0 ± 2.8	112.2 ±21.0
100 nM	7	181.8 ±42.9	28.4 ± 8.9	58.7 ±14.4	18.3 ± 3.4	67.5 ±23.6**
1 µM	7	149.9 ±24.6	27.4 ± 6.5	47.2 ±12.7	15.9 ± 3.8	65.7 ± 8.9***
10 µM	9	171.4 ±28.8	35.5 ± 7.9	55.6 +15.0	21.5 ± 5.6	63.2 ±11.1***
100 µM	6	163.5 ±39.4	36.2 ±13.0	70.3 ±19.0	22.5 ±10.9	46.7 ±12.5***
1 mM	6	114.2 ±24.7	36.6 ± 7.6	69.6 ±14.8	21.3 ± 4.3	52.7 ±18.9***

Values given are $\bar{x}$ ±S.E.M.; units fmol/mg/min.

Statistical comparisons were made with control (no inhibitor) using analysis of variance.

Significance levels: $^{*}0.05 > p > 0.01$

$^{**}0.01 > p > 0.001$

$^{***}0.001 > p$

Table 4.4.1.1. (c). Breakdown of ^{3}H LE by striatal membranes: effects of SQ24994 and of thiorphan.

Concentration	n	LE degraded	YGGF formed	YGG formed	YG formed	Y formed
Control	9	208.8 ± 35.3	32.2 ± 8.7	48.2 ± 12.2	18.0 ± 2.8	112.2 ± 21.0
SQ 10 µM	5	136.2 ± 27.5	11.7 ± 3.0*	18.8 ± 9.0	14.5 ± 2.5	96.8 ± 23.1
Th 10 µM	5	108.0 ± 33.4	13.2 ± 3.8*	8.5 ± 2.2	12.0 ± 2.5	80.0 ± 27.2

Statistical comparisons were made with control (no inhibitor) using analysis of variance.

Significance levels: $^{*}0.05 > p > 0.01$

Table 4.4.1.1. (d). Breakdown of ^{3}H LE by striatal membranes: effects of SQ 24994 in the presence of 100 μM bestatin.

Concentration	n	LE degraded	YGGF formed	YGG formed	YG formed	Y formed
Control	9	208.8 ± 35.3	32.2 ± 8.7	48.2 ± 12.2	18.0 ± 2.8	112.2 ± 21.0
100 pM	5	106.7 ± 25.7**	9.2 ± 3.1	55.8 ± 24.1	16.2 ± 3.4	23.3 ± 14.0***
1 nm	5	91.3 ± 29.0**	14.1 ± 5.8	56.6 ± 22.7	13.0 ± 2.0	10.3 ± 1.1***
10 nm	5	107.2 ± 54.7**	16.5 ± 6.1	66.0 ± 38.9	17.3 ± 8.1	13.2 ± 9.6***
100 mM	5	67.1 ± 19.2***	6.8 ± 0.7	36.2 ± 17.1	12.7 ± 3.5	9.7 ± 1.8***
1 μM	5	42.8 ± 7.4***	8.6 ± 2.5	9.7 ± 2.2	11.0 ± 4.1	8.0 ± 1.7***
10 μM	5	39.1 ± 7.2***	7.8 ± 2.2**	7.8 ± 3.2*	7.9 ± 3.3	8.2 ± 1.4***

Statistical comparisons were made with control (no inhibitor) using analysis of variance.

Significance levels: * $0.05 > p > 0.01$

** $0.01 > p > 0.001$

*** $0.001 > p$

Table 4.4.1.1. (e). Breakdown of ^{3}H LE by striatal membranes: effects of thiorphan in the presence of 100 μM bestatin.

Concentration	n	LE degraded	YGGF formed	YGG formed	YG formed	Y formed
Control	9	208.8 ± 35.3	32.2 ± 8.7	48.2 ± 12.2	18.0 ± 2.8	112.2 ± 21.0
100 pM	5	90.5 ± 33.5**	10.2 ± 3.4	51.1 ± 21.3	11.5 ± 2.6	8.8 ± 3.0***
1 nM	5	76.0 ± 26.3***	9.9 ± 2.9	44.0 ± 21.1	10.5 ± 2.1	7.0 ± 3.4***
10 nM	5	65.2 ± 33.4**	9.0 ± 2.9	39.1 ± 22.2	11.4 ± 3.7	7.1 ± 2.5***
100 nM	5	80.6 ± 26.3***	11.1 ± 3.1	45.5 ± 17.5	13.9 ± 1.8	7.5 ± 5.4***
1 μM	5	39.0 ± 13.8***	8.0 ± 1.9	12.6 ± 4.6	11.7 ± 2.6	7.8 ± 2.7***
10 μM	4	46.3 ± 9.4***	12.8 ± 3.6*	6.2 ± 3.5	10.2 ± 1.8	21.6 ± 8.3***

Statistical comparisons were made with control (no inhibitor) using analysis of variance.

Significance levels: * $0.05 > p > 0.01$

** $0.01 > p > 0.001$

*** $0.001 > p$

TABLE 4.4.1.2. (a)

BREAKDOWN OF LE BY STRIATAL MEMBRANES : SUMMARY OF FURTHER STATISTICAL COMPARISONS.

(a)

EFFECT COMPARED TO 1 mM BESTATIN

TREATMENT	n	LE DEGRADED	YGGF FORMED	YGG FORMED	YG FORMED	Y FORMED
PUROMYCIN 1 mM	6		INCREASE **	INCREASE *		INCREASE **
SQ 24,994 10 μM	5					INCREASE ***
BESTATIN 100 μM SQ 24994 10 μM	5					
THIORPHAN 10 μM	5					INCREASE ***
BESTATIN 100 μM + THIORPHAN 10 μM	4					

Significance levels : * $0.05 > p > 0.01$

** $0.01 > p > 0.001$

*** $0.001 > p$

In the above table and in tables 4.4.1.2(b) to (g), the absence of comment in a box means that there was no significant difference between treatments, i.e., $p > 0.05$.

TABLE 4.4.1.2. (b) BREAKDOWN OF LE BY STRIATAL MEMBRANES : SUMMARY OF FURTHER STATISTICAL COMPARISONS.

(b) EFFECT COMPARED TO 100 µM BESTATIN (n = 8)

TREATMENT	n	LE DEGRADED	YGGF FORMED	YGG FORMED	YG FORMED	Y FORMED
PUROMYCIN 100 µM	6		INCREASE **			INCREASE *
SQ 24,994 10 µM	5					INCREASE ***
BESTATIN 100 µM + SQ 24,994 10 µM	5			DECREASE *		
THIORPHAN 10 µM	5			DECREASE *		INCREASE ***
BESTATIN 100 µM + THIORPHAN 10 µM	4			DECREASE *		

Significance levels : * 0·05 p 0.01

** $0.01 > p > 0.001$

*** $0.001 > p$

TABLE 4.4.1.2. (c) BREAKDOWN OF LE BY STRIATAL MEMBRANES :
SUMMARY OF FURTHER STATISTICAL COMPARISONS.

(c) EFFECT COMPARED TO 10 µM BESTATIN (n = 8)

TREATMENT	n	LE DEGRADED	YGGF FORMED	YGG FORMED	YG FORMED	Y FORMED
PUROMYCIN 10 µM	9		INCREASED *			INCREASE ***

Significance levels :
* $0.05 > p > 0.01$
** $0.01 > p > 0.001$
*** $0.001 > p$

TABLE 4.4.1.2. (d) BREAKDOWN OF LE BY STRIATAL MEMBRANES :
SUMMARY OF FURTHER STATISTICAL COMPARISONS.

(d) EFFECT COMPARED TO PUROMYCIN 1 mM (n = 6)

TREATMENT	n	LE DEGRADED	YGGF FORMED	YGG FORMED	YG FORMED	Y FORMED
SQ 24,994 10 µM	5		DECREASE **	DECREASE *		INCREASE *
BESTATIN 100 µM + SQ 24,994 10 µM	5		DECREASE **	DECREASE **		DECREASE *
THIORPHAN 10 µM	5		DECREASE **	DECREASE **		
BESTATIN 100 µM + THIORPHAN 10 µM	4		DECREASE *	DECREASE **		

Significance levels :
* $0.05 > p > 0.01$
** $0.01 > p > 0.001$
*** $0.001 > p$

TABLE 4.4.1.2. (e) BREAKDOWN OF LE BY STRIATAL MEMBRANES : SUMMARY OF FURTHER STATISTICAL COMPARISONS.

(e) EFFECT COMPARED TO PUROMYCIN 100 µM (n = 6)

TREATMENT	n	LE DEGRADED	YGGF FORMED	YGG FORMED	YG FORMED	Y FORMED
SQ 24,994 10 µM	5		DECREASE **	DECREASE *		INCREASE **
BESTATIN 100 µM + SQ 24994 10 µM	5	DECREASE **	DECREASE **	DECREASE **		DECREASE *
THIORPHAN 10 µM	5		DECREASE **	DECREASE **		INCREASE *
BESTATIN 100 µM + THIORPHAN 10 µM	4	DECREASE **	DECREASE *	DECREASE **		

Significance levels :
- * $0.05 > p > 0.01$
- ** $0.01 > p > 0.001$
- *** $0.001 > p$

TABLES 4.4.1.2. (f) AND (g). BREAKDOWN OF LE BY STRIATAL MEMBRANES :
SUMMARY OF FURTHER STATISTICAL COMPARISONS.

(f) EFFECT COMPARED TO SQ 24994 10 µM (n = 5)

TREATMENT	n	LE DEGRADED	YGGF FORMED	YGG FORMED	YG FORMED	Y FORMED
THIORPHAN	5					
BESTATIN 100 µM + SQ 24994 10 µM	5	DECREASE *				DECREASE ***
BESTATIN 100 µM + THIORPHAN 10 µM	4					DECREASE ***

(g) EFFECT COMPARED TO THIORPHAN 10 µM (n = 5)

TREATMENT	n	LE DEGRADED	YGGF FORMED	YGG FORMED	YG FORMED	Y FORMED
BESTATIN 100 µM + SQ 24994 10 µM	5					DECREASE ***
BESTATIN 100 µM + THIORPHAN 10 µM	4					DECREASE **

Significance levels : * $0.05 > p > 0.01$
** $0.01 > p > 0.001$
*** $0.001 > p$

4.4.2. BREAKDOWN OF LE BY STRIATAL SLICES.

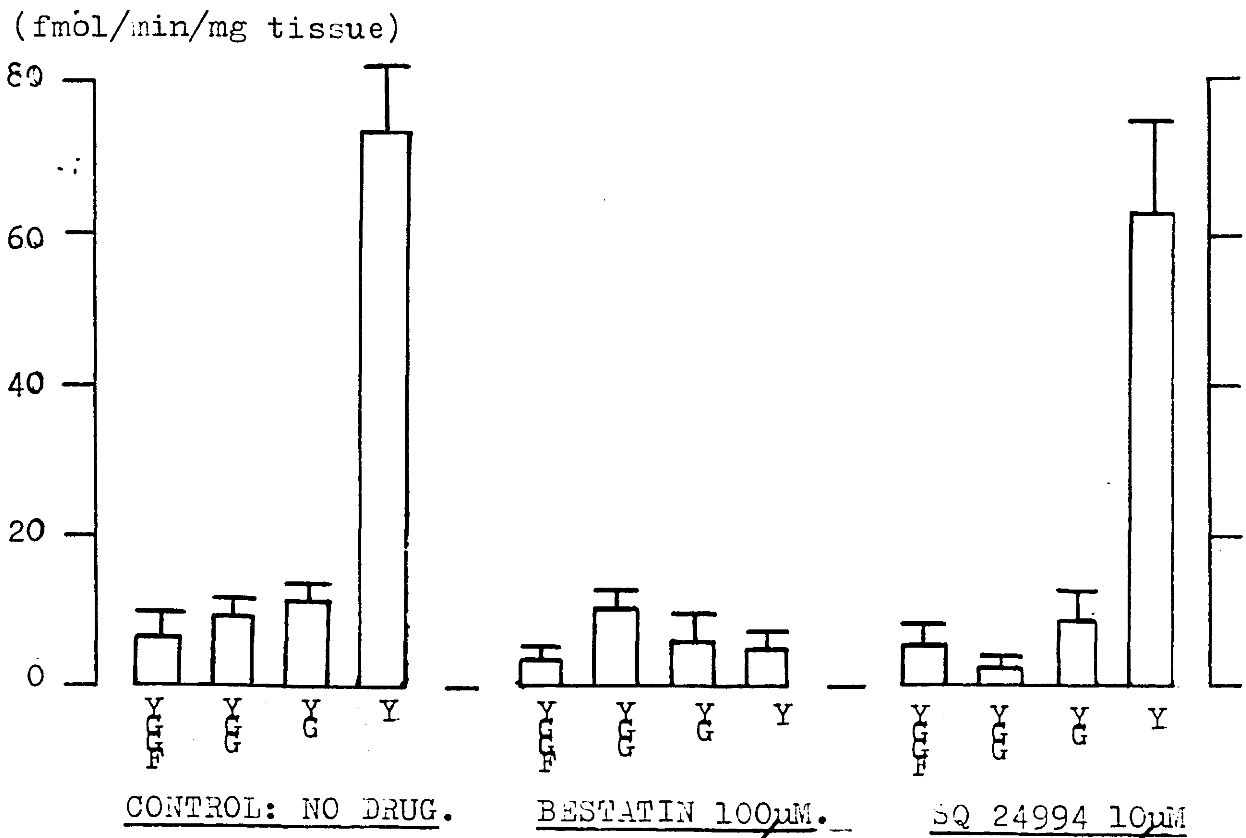
^{3}H LE at a concentration of 8 nM was incubated for 15 min with rat corpus striatum slices (section 34) and the labelled metabolites separated (sections 3.6 and 3.7) and quantified (section 3.8). Results were calculated as described in section 3.9 . The data has been presented as a series of histograms (figure 4.4.2.1.) and as a table giving rates of production formation averaged over 15 min (table 4.4.2.1.), in which drug effects were statistically compared with control. Further statistical comparisons have been presented in tables 4.4.2.2.(a) and (b).

Figure 4.4.1.1. shows that in the absence of inhibitors the major breakdown product was tyrosine. The amounts of the other three products were low and did not differ significantly from one another. A comparison of the "control" histogram with the "tissue removed" histogram (in the latter experiments LE had been incubated in saline in which tissue slices had previously been incubated) shows that a high level of enzyme activity was present in the incubation medium: the pattern of product formation differs, in that less Tyr-Gly-Gly was formed (table 4.4.2.1.), which may be evidence that the Tyr-Gly-Gly-forming peptidase activity was the most closely associated with the tissue slices. The activity in the medium could only have come from the tissue slices, suggesting that tissue damage had occurred - this possibility is discussed in section 5.2.3 .

A 100 μM concentration of bestatin inhibited the aminopeptidase activity very effectively, judging by the reduction in Tyr formation (fig. 4.4.2.1.), while SQ24994 had little or no effect on Tyr formation but significantly reduced the amount of Tyr-Gly-Gly present at the end of the incubation (table 4.4.2.1.). The different inhibitory specificities of these two compounds appear to be parallel in the slice and membrane preparations. A combination of the two substances was very effective in reducing LE breakdown, and gave LE significantly greater protection than did SQ24994 alone, but not bestatin alone. Using the more detailed information obtained from the experiments on LE degradation using striatal membranes (section 4.4.1.1.) conclusions may be drawn about the types of peptidases active in the striatal slice incubations (section 5.2.3.). The suitabilities of the

two types of striatal preparation as models of enkephalin breakdown in the intact tissue are compared in section 5.2.4.

Figure 4.4.2.1. Products of Leucine Enkephalin degradation by a striatal slice preparation.



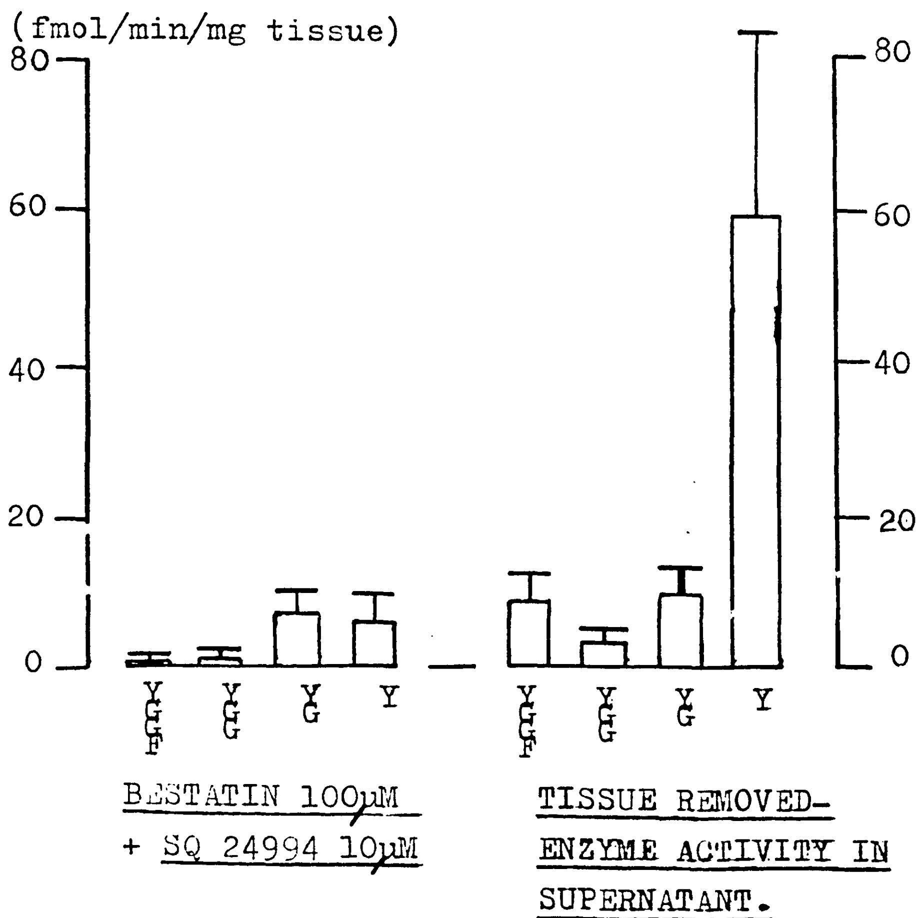


TABLE 4.4.2.1. BREAKDOWN OF LE BY STRIATAL SLICES : EFFECTS OF SQ 24994 AND BESTATIN.

TREATMENT	n	LE DEGRADED	YGGF FORMED	YGG FORMED	YG FORMED	Y FORMED
CONTROL	6	90.5 ± 11.6	6.5 ± 2.9	9.2 ± 1.7	12.2 ± 1.4	73.0 ± 9.4
BESTATIN 100 µM	6	20.9 ± 6.4 **	2.5 ± 1.2	9.6 ± 2.4	5.4 ± 4.3	4.6 ± 2.4 **
SQ 24,994 10 µM	7	70.4 ± 19.0	5.1 ± 2.6	2.7 ± 1.4 *	8.5 ± 4.4	62.6 ± 12.4
BESTATIN 100 µM + SQ 10 µM	7	11.1 ± 3.5 **	0.7 ± 1.0	0.7 ± 1.4 **	6.5 ± 3.3	5.4 ± 3.6 **
TISSUE REMOVED	6	73.1 ± 31.1	8.5 ± 3.8	2.8 ± 1.4 *	9.3 ± 4.0	58.8 ± 25.4

Statistical comparisons were made with control (no inhibitor) using analysis of variance.

Significance levels : * $0.05 > p > 0.01$

** $0.01 > p > 0.001$

"Values given are S.E.M.; units fmol/mg/min."

TABLE 4.4.2.2. BREAKDOWN OF LE BY STRIATAL SLICES : FURTHER STATISTICAL COMPARISONS.

(a) EFFECT COMPARED TO BESTATIN 100 µM (n = 6)

TREATMENT	n	LE DEGRADED	YGGF FORMED	YGG FORMED	YG FORMED	Y FORMED
SQ 24994 10 µM	7			DECREASE **		INCREASE *
BESTATIN 100 µM + SQ 24994 10 µM	7			DECREASE ***		

(b) EFFECT COMPARED TO SQ 24994 10 µM (n = 7)

TREATMENT	n	LE DEGRADED	YGGF FORMED	YGG FORMED	YG FORMED	Y FORMED
BESTATIN 100 µM + SQ 24994 10 µM	7	DECREASE *				DECREASE **

Significance levels : * $0.05 > p > 0.01$

** $0.01 > p > 0.001$

*** $0.001 > p$

4.5. EFFECTS OF PEPTIDASE INHIBITORS ON THE ENDOGENOUS ENKEPHALINS IN STRIATAL SLICES.

Following the demonstration (section 4.4.2.1.) that bestatin and SQ24994 were potent inhibitors of enzymes which might be involved in the physiological inactivation of enkephalins, experiments were carried out to determine whether these compounds affected the content of naturally-occurring ME and LE in the striatal slice preparation.

4.5.1. VARIATIONS IN ENKEPHALIN AND POTASSIUM CONTENTS WITH TIME; LEAKAGE OF LACTATE DEHYDROGENASE ACTIVITY.

An initial series of experiments measured the changes in ME and LE during 1 h. superfusion of striatal slices (section 3.10).

The endogenous enkephalins were extracted from the slices and purified using Porapak Q and high performance liquid chromatography (section 3.5) and quantified using ELISA (section 3.15) - the results are presented in table 4.5.1.1.

The metabolic viability of the slice preparation (section 3.1) was examined by measuring the retention of the initial potassium content of the tissues using flame photometry (section 3.16) while an attempt was made to assess the degree of mechanical damage to the tissues by detecting leakage of the soluble cytoplasmic "marker" enzyme, lactate dehydrogenase, into the superfusion medium (section 3.17).

The quantity of striatal tissue was determined by the Lowry method (section 3.18). It was found that the initial potassium content of the tissue was maintained during 1 h superfusion (table 4.5.1.2.), indicating the ability of the tissue to synthesize ATP and perform metabolic work.

Lactate dehydrogenase (LDH) was detected in the superfusion medium throughout the experiment (table 4.5.1.3a), but the amount lost (table 4.5.1.3b) was considered low compared to the total amount of enzyme in the tissue (section 5.3.1.); the LDH activity of successive 5 min fractions of superfusate was uniform for 1 h (table 4.5.1.3a).

During this period of incubation, the total enkephalin content of the slices apparently declined, but not to a statistically significant extent (table 4.5.1.1.). Enkephalin might be lost from the tissues by spontaneous release, by leakage from damaged cells, or by enzymic degradation within the tissues (these mechanisms are discussed in section 5.3.1.).

TABLE 4.5.1.1. SUPERFUSION OF CORPUS STRIATUM SLICES :

VARIATION OF ENKEPHALIN CONTENT WITH TIME.

Units are pmol/mg; values are given as MEAN ± S.E.M.

Number of replicates = 5 at each time point.

	INCUBATION TIME / min.		
	0	15	60
MET ENKEPHALIN	1.53 ± 0.29	1.22 ± 0.19	1.07 ± 0.16
LEU ENKEPHALIN	0.26 ± 0.05	0.43 ± 0.19	0.21 ± 0.05
TOTAL ENKEPHALINS	1.79 ± 0.31	1.65 ± 0.32	1.28 ± 0.17
RATIO MET ENK: LEU ENK	7.32 ± 2.47	5.23 ± 1.67	6.14 ± 1.42

No differences statistically significant at $p = 0.05$ were detected using single-factor anovar (2 tailed test).

TABLE 4.5.1.2. SUPERFUSION OF CORPUS STRIATUM SLICES : VARIATION OF POTASSIUM CONTENT WITH TIME.

	INCUBATION TIME / min.		
	0	15	60
POTASSIUM ION CONCENTRATION	0.60 ± 0.04	0.65 ± 0.06	0.59 ± 0.03

ALL VALUES ARE ± S.E.M., UNITS µmol/mg protein

NUMBER OF REPLICATES = 5 AT EACH TIME POINT.

NO SIGNIFICANT DIFFERENCES (AT $p = 0.05$)WERE DETECTED USING SINGLE-FACTOR ANOVAR (2 TAILED TEST).

TABLE 4.5.1.3. SUPERFUSION OF CORPUS STRIATUM SLICES : APPEARANCE OF LACTATE DEHYDROGENASE ACTIVITY IN SUPERFUSION MEDIUM.

(a) TOTAL AMOUNTS OF LDH LEAKED DURING SUCCESSIVE 5 min PERIODS

ONE "UNIT" OF ENZYME ACTIVITY 1 μmol NADH OXIDISED / min.

INCUBATION TIME / min	n	LDH ACTIVITY (UNITS x 10^{-4} /5 min/mg
5	7	9.5 ± 2.0
10	6	12.1 ± 2.3
15	8	14.9 ± 3.7
20	4	12.2 ± 1.5
25	4	9.3 ± 2.8
30	4	16.2 ± 4.2
35	4	10.7 ± 0.6
40	4	16.4 ± 6.9
45	4	7.2 ± 2.6
50	4	13.1 ± 3.7
55	4	10.6 ± 6.7
60	4	9.4 ± 3.7

NO STATISTICALLY SIGNIFICANT DIFFERENCES COULD BE DETECTED BETWEEN THE RATE OF LEAKAGE DURING ANY ONE 5 min PERIOD AND THE OVERALL MEAN RATE. VALUES GIVEN AS $\bar{x}$ ± S.E.M.

(b) TOTAL LDH LEAKED DURING 1 hour.

INCUBATION TIME / min	n	LDH ACTIVITY (UNITS / hr / mg)
60	4	10.6 ± 3.1

4.5.2. SUPERFUSION OF STRIATAL SLICES: EFFECT OF ENZYME INHIBITORS.

Slices of rat corpus striatum were superfused as described in section 311. The superfusion medium contained drugs which had been found (sections 4.4.1, 4.4.2) to inhibit striatal enzymes breaking down enkephalin.

After 1 hour superfusion, the slices were extracted (3.13), the enkephalins purified by HPLC (3.14) and the content of endogenous enkephalins assayed by ELISA (3.15). The protein content of the slices was determined by the Lowry procedure (3.18). The results are presented in table 4.5.2.

Table 4.5.2. Superfusion of corpus striatum slices: effect of enzyme inhibitors on endogenous enkephalin content.

	Control	Bestatin 100 µM	SQ 24994 10 µM	Bestatin 100 µM + SQ 24994 10 µM
MET Enkephalin	6.02 ±0.76	5.74 ±1.17	5.30 ±0.06	5.27 ±0.50
LEU Enkephalin	2.42 ±0.67	1.56 ±0.41	1.47 ±0.41	1.94 ±0.43
Total Enkephalin	8.44 ±1.34	7.30 ±1.31	6.77 ±0.43	7.21 ±0.47
Ratio MET Enk : LEU Enk	3.10 ±0.74	4.61 ±1.24	4.61 ±1.22	3.28 ±0.88

All values are $\bar{x}$ ±S.E.M.; Units pmol/min.

Number of replicates = 4 for each treatment.

No statistically significant differences (at $p = 0.05$) were detected using single factor anovar (2-tailed test).

4.6. ADMINISTRATION OF SQ24994 *in vivo*.

Inhibitors of enkephalin breakdown have been shown to exert antinociceptive effects in certain animal models (section 1.6) and may be the prototype compounds of a new class of therapeutic analgesics (section 1.5). The ability of these substances to attenuate perception of noxious stimuli may be a result of the accumulation of enkephalins at apioid receptor sites. If this is the case, it might be possible to detect an increase in the total enkephalin content of the brain of treated animals, consequent on inhibition of the enzymes responsible for inactivating the enkephalins of the intact animal. The novel peptidase inhibitor SQ24994 was administered to rats by intra-cerebro-ventricular injection, as described in section 3.12 . Control rats were injected with vehicle alone. 30 minutes later the enkephalin contents of the brains (minus cerebella) of treated and control animals was determined by ELISA (section 3.15) following HPLC purification (section 3.14).

Table 4.6 shows that both ME and LE were elevated in the brains of treated rats, compared to controls. This suggests that an SQ24994-sensitive enzyme may degrade enkephalins *in vivo* . This possibility is discussed in section 5.4 .

4.6. ADMINISTRATION OF SQ 24994 IN VIVO

The novel enzyme inhibitor SQ 24994 was administered to rats by intra-cerebro-ventricular injection (3.12) and after 30 min. drug treatment, the enkephalin content of the brains of treated and control animals was determined by ELISA (3.15) following HPLC purification (3.14) The results are shown in table 4.6.

Table 4.6. ME and LE content of rat brain, 30 min after i.c.v. SQ 24994 or saline.

Treatment	n	ME	LE	Ratio ME/LE
Control		0.42 $\pm$0.06	0.30 $\pm$0.07	1.70 $\pm$0.35
SQ 24994	6	0.56 $\pm$0.12*	0.47 $\pm$0.05*	1.25 $\pm$0.26

All values given as $\bar{x}$ $\pm$S.E.M., Units pmol/mg.

*Statistically significant difference from control ($p \leqslant 0.05$; Student's t-test)

4.7 EFFECTS OF SQ 24994 ON MOUSE VAS DEFERENS BIOASSAY.

Addition of 6.67 µM SQ 24994 did not affect the twitch amplitude (fig. 4.7 a). Addition of SQ 24994 shifted the dose response curves for LE to the left (fig. 4.7 b). The increased sensitivity to LE is reflected in the reduced IC 50 values (table 4.7).

TABLE 4.7

I.C. 50 VALUES OF LE ON M.V.D. BIOASSAY

UNITS: nM. VALUES ARE MEAN ± S.E.M.; n = 5

	IC 50/nM
No SQ 24994	10.3 ± 1.8
6.67 µM SQ 24994	23.3 ± 6.5
MEAN DIFFERENCE	18.5 ± 2.9
MEAN RATIO	3.0 ± 0.8

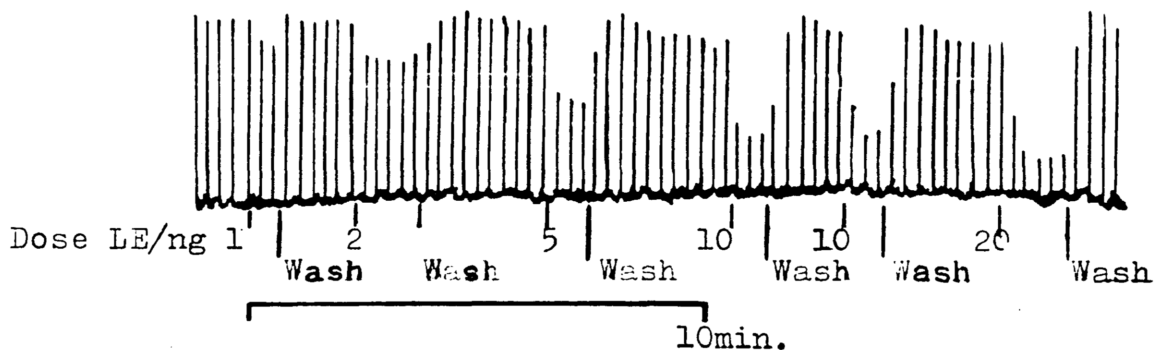
PAIRED - SAMPLE TEST GAVE STUDENT'S t = 6.35

$0.01 > p > 0.001$

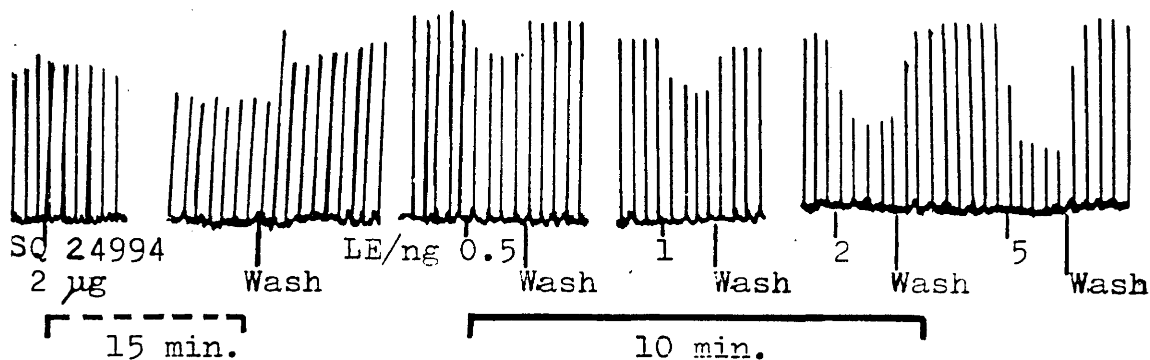
i.e. SQ 24994 HAS SIGNIFICANTLY LOWERED IC 50.

Figure 4.7.(a). Effects of **SQ 24994** on the response to Leucine Enkephalin of the mouse vas deferens bioassay.

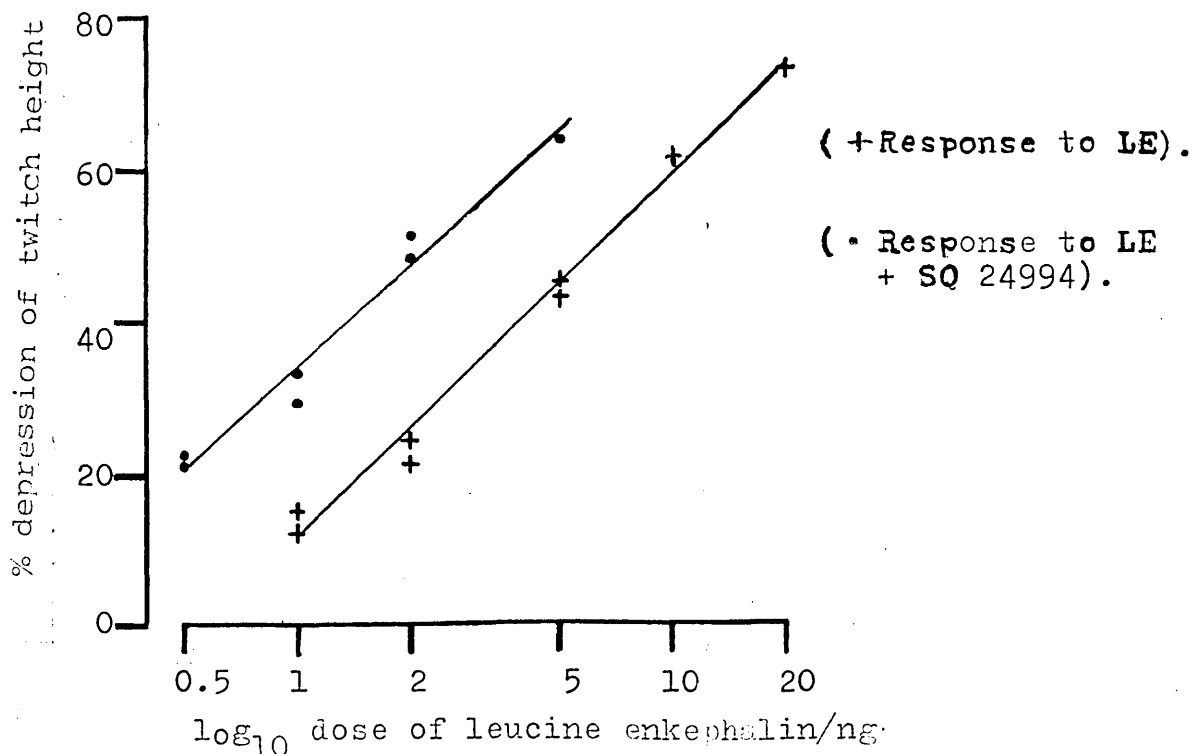
Leucine Enkephalin depresses the twitch response of the tissue to electrical field stimulation (section 3.   ), probably by inhibiting the evoked release of noradrenalin (Hughes et al, 1975).



The compound **SQ** 24994 does not itself affect twitch amplitude, but potentiates the inhibitory effect of LE.



The $\log_{10}$dose versus response graph is shifted to the left. (Lines fitted by linear regression: r=0.99: n=2.)



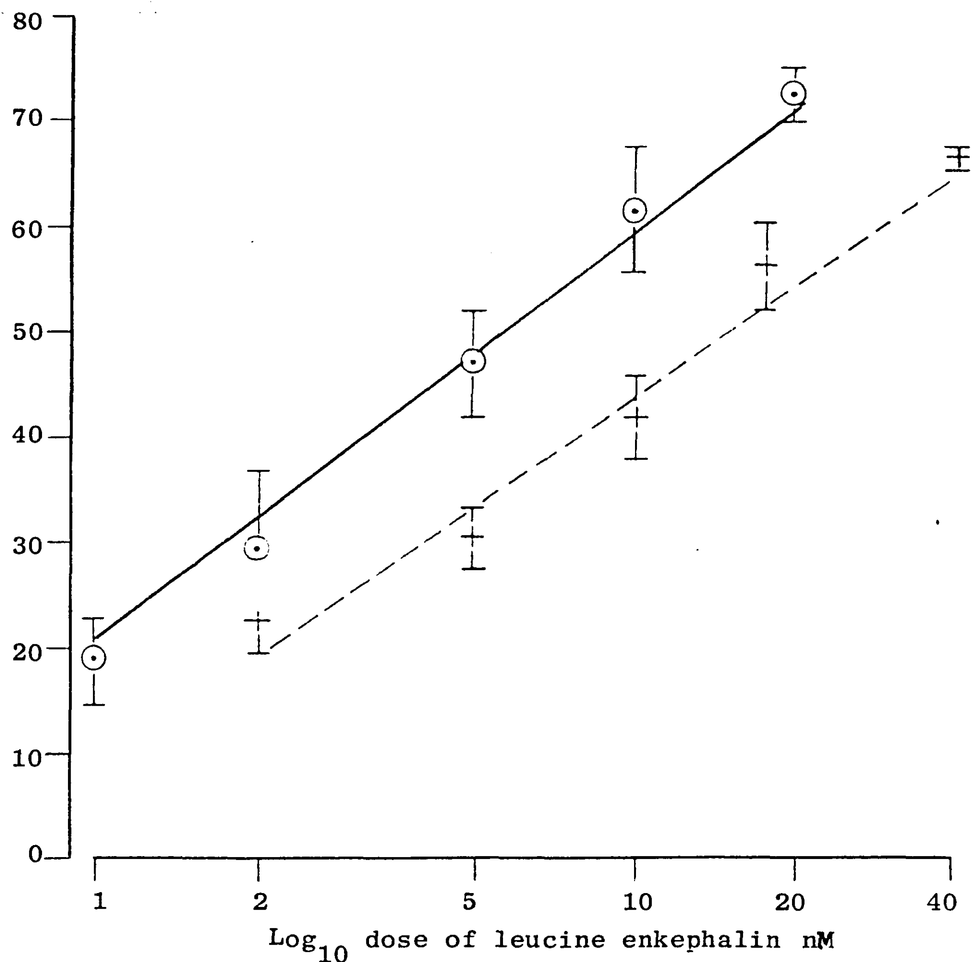


Fig. 4.7(b): Effect of SQ24994 on the response to LE of the mouse vas deferens bioassay.

+ Response of untreated tissue to LE.

⊙ Response after incubation with 6 µM SQ24994.

Lines were fitted by linear regression; $n = 5$; $p<0.05$.

D I S C U S S I O N

5.1. PURIFICATION OF TRITIATED METABOLITES.

The trace shows that the buffer system used did not separate Tyr, Tyr-Gly and Tyr-Gly-Gly. To increase separation with a reverse phase column, it is usual to use a more hydrophilic solvent, so that substances are retained longer on the column. However, these three products have a 10 min. elution time, so that this strategy would have increased the retention of LE by an unacceptable amount. It was anticipated that the separation of these substances on the basis of differences in hydrophobicity would be an insuperable problem using this column, and thin-layer chromatography was tested as a separation technique.

The solvent mixture finally selected (Malfroy et al, 1979) separated all the possible labelled metabolites, provided that inorganic salts were first removed from the sample.

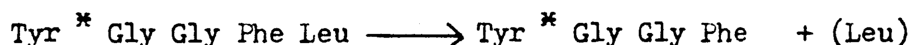
The Dowex method for de-salting was shown to give equivalent recoveries of LE and Y at low concentrations.

The combination of ion exchange and TLC was found to have the advantage over the hydrophobic resins used by most workers (Hudgin et al, 1981; De la Baume et al, 1982, 1983) that all products were separated in one step.

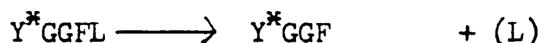
5.2.1. METABOLISM OF LE BY BRAIN TISSUE PREPARATIONS.

LE may be enzymically hydrolysed at any of its four peptide bonds; any such cleavage destroys biological activity. When LE, tritium-labelled at its N-terminal Tyr (Y) residue, is incubated with preparations of brain tissues, the presence of specific inactivating enzymes may be indicated by the formation of labelled products (reactions 1 to 4). Obviously only those radioactive fragments containing the tritiated Tyr residue (denoted Y *) will be detected by scintillation counting.

1. Carboxypeptidase activity;



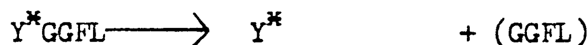
or, using the single-letter code for amino acids;



2. Endopeptidase, or dipeptidyl carboxypeptidase activity;

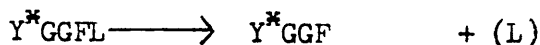
3. Endopeptidase, or dipeptidyl aminopeptidase activity;

4. Aminopeptidase activity;



Some of the labelled products may be formed by more than one route, for example :

sequential activity of carboxypeptidase;



5. $Y^{*}GGF \longrightarrow Y^{*}GG \quad + (F)$

6. $Y^{*}GG \longrightarrow Y^{*}G \quad + (G)$

on the other hand, a product of the primary cleavage of LE might be attacked by a second enzyme of a different type;

e.g., aminopeptidase following carboxypeptidase;

7. $Y^{*}GGF \longrightarrow Y^{*} \quad + (GGF)$

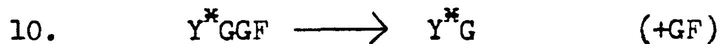
aminopeptidase following either an endopeptidase or a dipeptidyl carboxypeptidase;

8. $Y^{*}GG \longrightarrow Y^{*} + (GG)$

dipeptidase cleaving the product of either endopeptidase or dipeptidyl aminopeptidase activity;

9. $Y^{*}G \longrightarrow Y^{*} + (G)$

or the following cleavage of the primary product of carboxypeptidase activity;


10. $Y^{*}GGF \longrightarrow Y^{*}G \quad (+GF)$

which might conceivably be carried out by an endopeptidase, a dipeptidyl carboxypeptidase or a dipeptidyl aminopeptidase. Furthermore, since only Y^{*} is necessarily immune to further enzymic hydrolysis, the disappearance of the primary cleavage products of LE may complicate the interpretation.

Throughout this series of experiments, a substrate of high specific radioactivity was used at a concentration of 8 nM. The values reported in the literature for the Km's of the enzymes regarded as important in enkephalin inactivation are all in the range from 1 μM to 1 mM.

Of these enzymes, enkephalinase has a Km of 20 - 30 μM for LE (Schwartz et al, 1980; Fournie-Zaluski et al, 1979) while the Km's of some aminopeptidases are of the same order; that purified by Hersh and McKelvy (1981) had a Km of 22 μM and Hudgin et al (1981) reported an overall Km of 15 μM for the aminopeptidases of striatal membranes. The Km for LE of angiotensin converting enzyme is probably about 100 μM (Benuck et al, 1981) although Schwartz et al quote a value of 1 mM (Schwartz et al, 1980).

Under these experimental conditions, substrate concentrations would be expected to limit the rate of reaction. In fact, since any peptidase presumably binds only one substrate molecule at a time, kinetics will probably approach the "first order" situation, so that the rate of reaction will be proportional to substrate concentration. Thus the rapid activity of one enzyme might reduce the availability

of substrate to another enzyme.

Enzyme inhibitors were introduced into the experiments with both slice and membrane preparations of striatum. The selective inhibition of one type of enzyme activity can clarify the relationships between products and enzymes.

5.2.2. BREAKDOWN OF LE BY STRIATAL MEMBRANES.

The membranes were prepared from the corpus striatum, a brain area with a high level of "enkephalinase" activity (Malfroy et al, 1979) and with a high concentration of enkephalins (reviewed by Cuello, 1983). This tissue was also chosen for the preparation of slices.

The formation of each of the four labelled products will be examined in turn, under various conditions of drug treatment; discussion will initially be limited to the experimental data, without using background information available from the literature.

Tyr-Gly-Gly-Phe (YGGF)

The only route of YGGF formation is LE cleavage by a carboxypeptidase (reaction 1). The amount of YGGF formed is significantly reduced by 10 µM SQ 24,994, 10 µM thiorphan, or 100 µM or 1 mM bestatin, but is unaffected by puromycin (tables 4.4.1.1. a,b,c). It is not possible to identify the metabolic fate of YGGF, since those compounds which reduce its formation also reduce the formation of the products which might result from its breakdown; the only information available from the data is that 1 mM puromycin substantially reduces Y formation, so that it is unlikely that most of the YGGF formed is broken down to Y.

Tyr-Gly-Gly (YGG).

In the absence of drugs YGG accounts for about 23% of total product formation (fig. 4.4.1.1. a). The results do not provide sufficient evidence to deem either of the two possible routes of YGG formation (reactions 2 and 5) more likely than the other.

If only one carboxypeptidase generated YGG from LE (scheme 5) it would seem likely that any compound inhibiting one cleavage step would also inhibit the other. Doses of bestatin of up to 100 µM suppress YGGF formation without reducing YGG levels (table 4.4.1.1 a).

In the presence of 100 µM bestatin, SQ 24,994 or thiorphan appear to reduce YGG content at doses of 100 µM or higher whereas YGGF formation is suppressed throughout. These observations perhaps favour the existence of two enzymes, one forming YGGF and one forming YGG, although the substrate for the latter enzyme is uncertain. If this deduction is correct then the reduction in YGG formation apparent with bestatin at 1 mM would be attributable to an inhibition of the formation of YGG from its immediate precursor (table 4.4.1.1. a also fig. 4.4.1.1.a).

YGG may itself be subject to enzymic degradation; that this is so, may be inferred from the apparent rise in YGG content in the presence of puromycin, or of bestatin at concentrations up to 100 µM (tables 4.4.1.1. a,b). Results discussed below show that these drugs are aminopeptidase inhibitors.

Tyr-Gly (YG).

There are three possible routes to YG (reactions 3, 6, 10). In contrast to the other three products, levels of YG were not significantly affected by any drug treatment, remaining fairly constant under conditions significantly depressing YGGF formation (100 µM bestatin, 10 µM SQ 24,994 or 10 µM thiorphan; tables 4.4.1.1. a,c), and in the presence of 100 µM bestatin plus 10 µM SQ 24,994 (table 4.4.1.1. d) where the contents of both YGGF and YGG are depressed, so that it is perhaps more likely that YG is formed directly from LE. The elimination of Y formation by 1 mM bestatin does not alter YG levels (table 4.1.1. a) so that dipeptidase activity is probably not significant in this system.

Tyr (Y).

Either SQ 24,994 or thiorphan at 10 µM will markedly reduce the levels of YGGF and YGG without significantly affecting Y formation (table 4.4.1.1. c) while YG is unlikely to be a major source of Y. Conversely, puromycin will significantly decrease Y formation at as

low a concentration as 100 nM, although the level of no other product is reduced (table 4.4.1.1. b).

Thus, while other substrates may contribute to free Y, an independent route probably exists, by which Y is formed directly from LE by aminopeptidase activity.

A more unified interpretation will now be attempted, of the effects of each drug treatment on routes of LE catabolism.

Puromycin.

At 1 mM, this compound significantly reduced the degradation of LE by the striatal membranes. The protection of LE is wholly attrubutable to an inhibition of aminopeptidase activity, which is statistically significant at a puromycin concentration of 100 nM. This action of puromycin has been amply documented (section 1.3.3.). The dose-response curve is of very shallow slope (fig. 4.4.1.1. d). A possible explanation is that more than one Y forming activity is present, and that one component is readily inhibited by puromycin, while another is refractory (Hersh, 1981).

The poor protection, by only 45%, of LE by puromycin is consistent with the observation of Patey et al (1981) that the drug afforded no protection to endogenous enkephalins released from brain slices. However, De la Baume et al (1983) found that puromycin had an IC 50 of 460 nM against the aminopeptidase activity of striatal membranes. This discrepancy may partly be explained by differences in experimental method; De la Baume et al did not wash the membranes following the initial centrifugation, while in this series of experiments the preparation was washed extensively and may have been less contaminated by cytoplasmic enzymes (section 1.3.1. d). It is also possible that the different buffers used in the two experiments may have influenced the responses of the enzymes.

It seems likely that the elevated YGG levels at the higher puromycin concentrations result from the inhibition by the drug of YGG break-

would suggest; the IC 50's of the compound were given as 3.5 nM and 140 nM against enkephalinase and ACE respectively. It is not impossible, therefore, that SQ 24,994 derives some part of its potency as an inhibitor of YGG formation from its inhibition of angiotensin converting enzyme. Both drugs significantly decreased the YGGF formation, i.e., both are carboxypeptidase inhibitors, and are of similar potencies. The development of this class of drug from carboxypeptidase inhibitors is described in section 1.5.6.; presumably there is some residual affinity for the carboxypeptidase active site, although the effects of the two compounds in these experiments suggest that their Ki's for carboxypeptidase are probably much higher than that of SQ 14603 (2-benzyl 3-mercapto-propanoic acid) against carboxypeptidase A; a value of 11 nM was quoted for the latter compound by Ondetti et al (1979). A carboxypeptidase degrading enkephalins would be expected to be of "A - type" specificity; the models of the active sites of carboxypeptidase A (fig. 1.5.6. a) and of enkephalinase (fig. 1.5.6.a) and of enkephalinase (fig. 1.5.6. c) would predict that the greater distance of the terminal carboxyl group from the aromatic moiety and the sulphydryl group in thiorphan than in SQ 14603 would diminish the potency of thiorphan as a carboxypeptidase inhibitor. It has been emphasized that the formation of YGGF raises the possibility that it may be the immediate precursor of at least some of the YGG formed in these experiments; the similar effects of SQ 24,994 and thiorphan on YGGF formation may in fact be part of the explanation of their effects on YGG levels.

Neither thiorphan nor SQ 24,994 had any significant effect on the levels of either YG or Y.

Bestatin.

Bestatin reduced LE breakdown more than any of the other drugs; the IC 50 was about 10 nM (table 4.4.1.1. a; fig. 4.4.1.1. b). Although

down by aminopeptidase. Since the true rate of YGG formation is unknown it is impossible to say whether puromycin affects this process; Fulcher et al (1982) maintain that puromycin inhibits "enkephalinase" activity, in a similar membrane preparation.

SQ 24,994; thiorphan.

Thiorphan is the classic enkephalinase inhibitor (Roques et al, 1980) and at 10 μM markedly diminished the YGG content of the incubations, as did SQ 24,994, although neither did so to a statistically significant extent (table 4.4.1.1. c). No significant differences could be detected between the effects of these two drugs, whether used alone or in combination with bestatin. Gordon et al (1983) reported that the L, L isomer of corresponding structural formula to SQ 24,994 had IC 50 1.34 μM versus purified "enkephalinase"; the D, L isomer had an IC 50 of 1.43 μM (this is the compound SQ 27,222). The compound SQ 24,994 used here is a mixture of the D, L and L, L isomers (fig. 5.2.2). Gordon et al found thiorphan had an IC 50 of 0.36 μM against enkephalinase; thus some difference in potencies might have been expected in these experiments. As the mechanism of YGG formation in this preparation is unknown it is possible that an enzyme (or enzymes) other than enkephalinase is being inhibited. The affinity of angiotensin converting enzyme for enkephalins is much lower than that of enkephalinase, and its contribution to YGG formation in this system should have been minimized by the use of a very low concentration of LE substrate, but its participation cannot be absolutely ruled out. The two isomers of which SQ 24,994 is composed are both better inhibitors of angiotensin converting enzyme than of enkephalinase; the IC 50's of the L, L and D, L isomers are 0.15 and 0.70 μM respectively. The IC 50 of thiorphan versus ACE is 0.86 μM (Gordon et al, 1983). However, Roques et al, (1983) reported that thiorphan was much more selective between these two enzymes than the data of Gordon et al

puromycin began to exert a significant inhibitory effect on aminopeptidase activity than did bestatin (table 4.4.1.1. b), bestatin was significantly more potent than puromycin over the range 10 µM to 1 mM (tables 4.4.1.2. a, b, c). In fact, the dose-response curves have quite different shapes. Two categories of aminopeptidase activity may be present, one of which is inhibited only by bestatin, the other being sensitive to both drugs. The puromycin sensitive species seems to make the lesser contribution to enkephalin degradation in this membrane preparation, in accordance with the results of De la Baume _et al_ (1982, 1983) but in contradiction to the findings of Hersh (1981).

Bestatin at concentrations of 100 µM or higher significantly reduces YGGF formation (table 4.4.1.1. a; fig. 4.4.1.1. e), presumably by inhibiting a carboxypeptidase; the data give the impression that the potency against carboxypeptidase of bestatin is probably lower than that of SQ 24,994 or of thiorphan.

It has been suggested above, that YGG is hydrolysed by an aminopeptidase sensitive to puromycin, which thus enhances the recovery of YGG. Although 1 mM bestatin abolishes Y formation, YGG levels appear to be lower than those in controls, and are significantly lower than YGG levels in the presence of 1 mM puromycin (table 4.4.1.2. a). This may imply that bestatin directly inhibits YGG formation. The reduced levels of YGG may be a secondary consequence of the reduced availability of YGGF; on the other hand, it cannot be ruled out that the compound inhibits YGG formation directly.

In section 1.3.3. a model was presented of the binding of bestatin to a zinc containing aminopeptidase (Cushman and Ondetti, 1981). The adjacent carbonyl and hydroxyl groups in bestatin may chelate zinc in a similar way to the binding of the hydroxamate to the zinc atom of thermolysin (Nishino and

Powers, 1979); as previously described, there may be close similarities between the active sites of thermolysin and enkephalinase. Hydroxamates are in fact potent enkephalinase inhibitors (Hudgkin et al, 1981) and can also inhibit aminopeptidases (Coletti-Previero et al, 1982). In fig. 5.2.2., the structures of bestatin and a potent hydroxamic - acid enkephalinase inhibitor are compared with those of thiorphan and SQ 24,994. It has in fact been reported elsewhere, that bestatin is a weak enkephalinase inhibitor (Hachisu et al, 1982) of IC 50, 590 µM; the fact that the relative potencies of a series of bestatin analogues as inhibitors of enkephalinase (Hachisu et al, 1982) and of aminopeptidase (Wagner and Dixon, 1981) are exactly the same (the correlation coefficient is greater than 0.9), may be evidence of some underlying resemblance between the active sites of these two enzymes. It is not surprising that the specificity of these inhibitors is less than absolute. When used at rather high concentrations, bestatin, SQ 24,994 and thiorphan do not have unique specificities; they have spectra of activity, which overlap owing to some limited similarity in the active sites of the enzymes. In this respect, it is interesting that captopril, developed as an inhibitor of angiotensin converting enzyme, is a reasonably potent inhibitor of leucine aminopeptidase (Cushman and Ondetti, 1981).

100 µM bestatin plus SQ 24,994 or thiorphan.

Either drug combination is highly effective at protecting LE from enzymic degradation, in agreement with the results of De la Baume et al (1983). Bestatin alone at 100 µM significantly reduces the formation of LE by about 50%, and the additional protection afforded by either of the other drugs seems to be at a maximum at concentrations of 1 µM or 10 µM, when LE breakdown is approximately 20% control level. The combination of 10 µM SQ 24,994 and 100 µM bestatin protects LE significantly better than 10 µM SQ 24,994

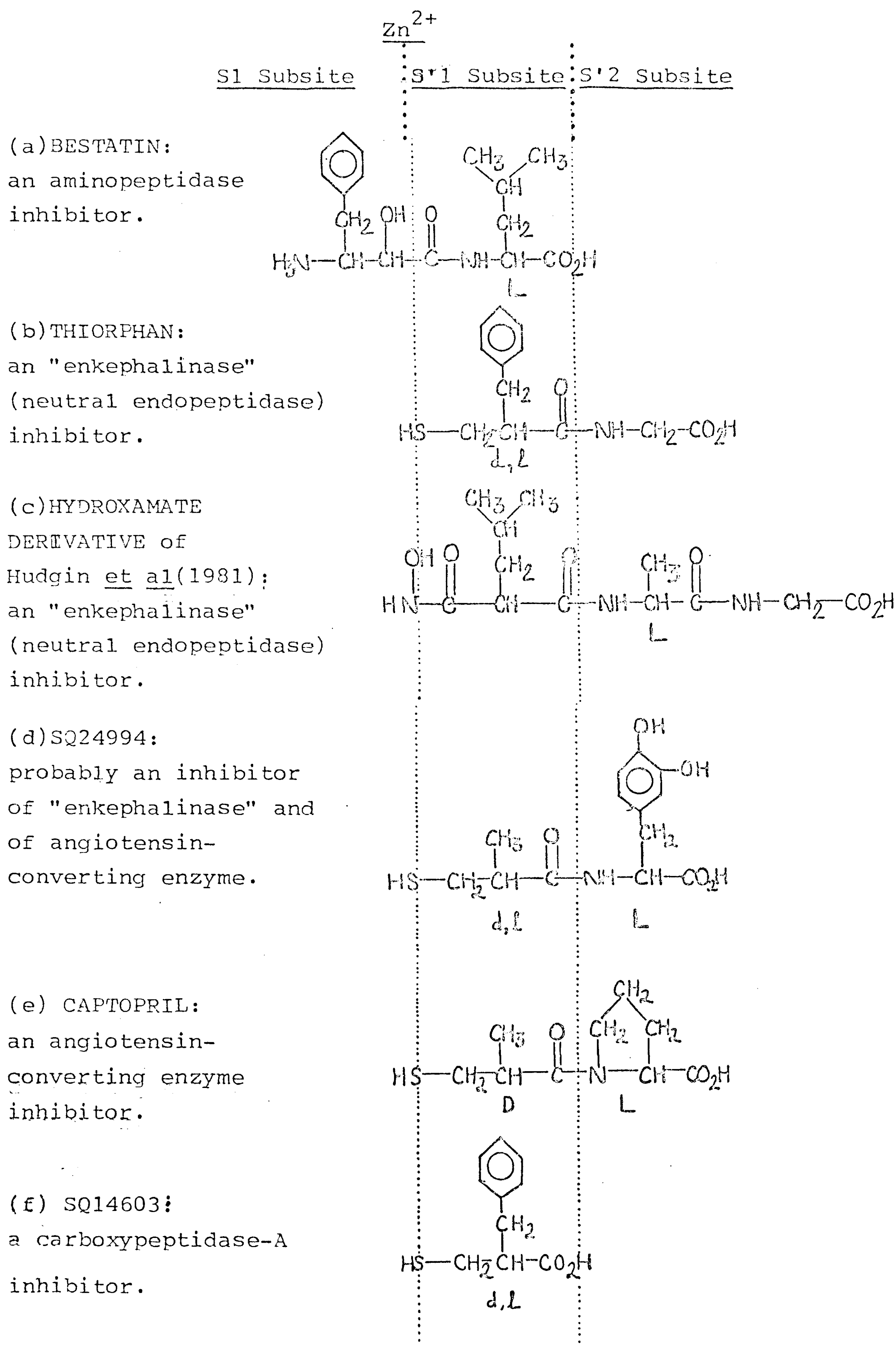


Figure 5.2.2. Comparison of the structures of inhibitors of enkephalin-degrading enzymes.

10 μM thiorphan is very effective at protecting LE from breakdown.

5.2.3. METABOLISM OF LE BY STRIATAL SLICES.

Control.

All four possible degradation products were found in the medium at the end of the incubation. It is striking that a very much higher proportion of LE is processed to Y in this slice preparation, than in the membrane preparation. In fact the overall pattern of products is different (fig. 4.4.2.2.), with YG the product next in quantity to Y.

"Tissue removed" incubation.

Products formed in this incubation have resulted from enzyme activity present in the medium bathing the slices. These enzymes will have been gained by the medium, from the tissue slices, during the pre-incubation stage (section 3.4). In the other incubations, the medium had been replaced immediately before the addition of LE substrate, whereas the "tissue removed" medium had accumulated enzyme activity for the full 15 min period, before substrate was added; the level of activity in the medium would therefore be expected to be higher than that present in the other incubations. The transfer of the slices to the wells of the plate used for the incubations may have caused some tissue damage, so that more enzyme may have leaked into the medium during the first 15 min incubation, when the solution was collected for the "no tissue" incubation, than during the second 15 min, when the slices were incubated with substrate.

The quantity of enzymes found in the "no tissue" incubation is probably an overestimate of that which leaked during the incubations of slices with substrate. Nevertheless, the "tissue removed" results should give a qualitative indication of the relative activities of different enzymes in the bathing medium.

The maintenance of a high internal potassium content, against a concentration gradient (table 4.5.1.2.) demonstrates that a high

proportion of the cells in the striatal slices possessed both intact plasma membranes, and the ability to carry out metabolic work, at the end of the 1 h. superfusion which preceded the experiment. Although there was a leakage from the slices into the medium of the soluble cytoplasmic enzyme, lactate dehydrogenase (table 4.5.1.3. a, b), the enzyme lost was probably a small fraction of the total tissue activity. These results give the impression that the tissues were very sensitive to handling and that the rate of leakage of cellular contents was increased during the incubations with tracer. It is implied above that soluble cytoplasmic enzymes contributed most of the enzyme activity in the medium, but it is conceivable that minute particles of membrane, bearing enzyme activity, may have become detached from the slices.

It is apparent that the very high level of aminopeptidase activity found in the control (no inhibitor) incubation was mainly operating in the bathing medium. The levels of soluble aminopeptidases in brain tissue are many times higher than the membrane bound activity (Lane et al, 1977; Hersh, 1981) and it appears that large amounts of this soluble enzyme have leaked from the tissue. The levels of both YGGF and YG in the "tissue removed" incubation are about the same as those formed in the presence of tissue. The formation of the various products by the membrane preparation is good evidence that the enzymes responsible are present bound to membranes in the tissue slices. However, soluble carboxypeptidases appear to exist within the slices. The origin of YG in the slice preparation is uncertain; it is probably formed directly from LE in the membrane preparation, by dipeptidyl aminopeptidase activity, such as the "enkephalinase B" of Gorenstein and Snyder (1979, 1980). It is not known whether enkephalinase B has a soluble, cytoplasmic form, but dipeptidyl aminopeptidases of other types are found in lysosomes (Kato et al, 1980).

Unlike the other three products the concentration of YGG is significantly lower in the "tissue removed" incubation than in control, demonstrating a close association of the YGG - forming activity with the tissue slices. This activity is thus distinct from the carboxypeptidase activity forming YGGF, at least that in the soluble phase.

100 µM bestatin.

Bestatin significantly protects LE from degradation, chiefly by inhibiting soluble aminopeptidases; this compound is presumably an inhibitor of both soluble and membrane bound forms of these enzymes, since Y formation is significantly decreased.

YGGF appears to be formed at a much lower rate in the presence of 100 uM bestatin, but the reduction was not statistically significant.

In this incubation, YGG was approximately at control levels, although aminopeptidase activity was strongly suppressed. The increase of YGG levels by puromycin in the membrane preparation suggested that YGG was a substrate for a membrane bound aminopeptidases. Possibly YGG is a poor substrate for the soluble aminopeptidases or perhaps bestatin is inhibiting the formation of YGG as well as its breakdown; this possibility was considered in the discussion of the experiments with striatal membranes. Alternatively, the reduction in YGG formation might have been a consequence of the reduction in YGGF formation.

10 µM SQ 24,994.

The YGG content of the incubation was significantly decreased by this drug. There was no significant effect on any other product. The lack of an effect on YGGF formation is striking in comparison with the statistically significant inhibition of carboxypeptidase activity in the membrane preparation. The soluble carboxypeptidase may be less readily inhibited than the particulate activity. There is no evidence that YGGF is a precursor of YGG in the slice incubations,

and a route of YGG formation directly from LE is more consistent with the data.

Since the YGG content was significantly reduced without affecting the formation of Y, it is reasonable to suppose that the routes to Y in this preparation involve substrates other than the tripeptide.

100 µM bestatin plus 10 µM SQ 24,994.

This drug combination significantly protected the exogenous LE from degradation; the total amount of LE broken down was reduced to about 12% of control values. These results would support the contention of De la Baume et al (1982,1983), that both aminopeptidase and YGG - forming activities are important in this preparation. However, the relative contributions of different enzyme activities differ from those reported by De la Baume et al, especially in the high ratio of aminopeptidase to YGG forming activities. In this system investigated by these workers the incubation medium was apparently free from enzyme activity. The absence of YGGF from the metabolites in their experiments can be explained by their use of Porapak Q chromatography. Consistent with their effects when used individually, the combination of the two inhibitors significantly reduces the formation of YGG and Y.

Breakdown of LE by striatal slices :

Summary.

1) The same types of LE degrading enzymes were present in the striatal slices as in the striatal membrane preparation.

2) A YGG forming activity is closely associated with the tissue slice. The evidence tends to favour the formation of YGG by an enzyme activity distinct from that forming YGGF.

3) In addition to the membrane bound forms of enzymes which presumably exist at the surfaces of the cells in the slices, soluble enzyme activity was found in the medium bathing the slices.

4) The major product of LE catabolism is Y, a high proportion of

which is probably cleaved directly from LE.

5.2.4. COMPARISON OF THE USE OF STRIATAL SLICES AND STRIATAL MEMBRANES.

Patey et al (1980), De la Baume et al (1982, 1983) and Chaillet et al (1983) have extensively studied the effects of puromycin, bestatin and thiorphan on both types of striatal preparation. This group of workers have claimed that chopped tissue is superior to a membrane preparation, since the particles of membrane may adsorb soluble cytoplasmic enzymes freed when cells are ruptured during homogenization. If this is the case, then it would be expected that cytoplasmic peptidases exuding from cells damaged during chopping would bind to the membranes of cells in the slice and would contribute to the total enzyme activity of the tissue, although media in which the chopped tissue had been incubated might show no residual enzyme activity. On the other hand, it is easy to wash membrane particles extremely thoroughly, by homogenization into large volumes of buffer, followed by centrifugation. Thus striatal slices are not necessarily freer from contamination than striatal membranes.

It has been described above, how the striatal slices used in the present study leaked enzyme activity into the bathing medium. Mainly because of this high enzyme activity in the medium, the amount of activity intrinsic to the slices is uncertain. The formation of products in the "tissue removed" samples was variable (table 4.4.2.1.) because some tissue slices leaked more enzyme than others. Variations in the enzyme activity in the medium probably caused most of the variability in the samples containing tissue slices.

The interpretation of the results of the work with striatal slices was based on that of the results from the membrane preparation; the results from slices are generally consistent with those obtained using membranes. However, the latter system was investigated in greater detail, and the data from the

membrane preparation has been given more weight in the ensuing discussion.

5.3.1. VARIATION WITH INCUBATION TIME OF THE ENKEPHALIN CONTENT OF SUPERFUSED STRIATAL SLICES.

Over the course of 1 h. there was an apparent decline in the enkephalin content of the slices, by about 30%, although the decrease was not statistically significant. (The least difference which would have been significant was 42%). The rate of loss of enkephalin from the slices was apparently uniform; because of the overall lack of statistical significance the fluctuations in ME or LE content seen on the graph are probably random. Several workers have reported that the enkephalin content of striatal slices is decreased by _in vitro_ superfusion. Osborne _et al_, (1978) observed a 15 - 20% loss of ME from rat striatal slices over 25 min. Bayon _et al_ (1978) found that 74% of ME and 56% of LE was lost from slices of rat globus pallidus superfused for 39 min., and Harsing _et al_ (1982) found that 67% of rat striatal ME disappeared during 75 min. superfusion.

Even in the absence of depolarizing stimuli there is a low rate of calcium - independent efflux of neurotransmitters from tissue slices or from synaptosomes _in vitro_. This phenomenon has invariably been observed in experiments on enkephalin release, but in no instance was this "spontaneous efflux" or "basal release" at a rate sufficient to account for more than a small fraction of the loss of tissue enkephalin - for example, Osborne _et al_ (1978) reported a release of 0.05% of tissue stores per min., and Iversen _et al_ (1978) and Harsing _et al_ (1982) reported basal leakage rates of 0.2% per min. and less than 0.1% per min. respectively.

The preparation of tissue slices inevitably damages many cells, the cytoplasm of which may leak out into the medium; chiefly at the beginning of the incubation period. Iversen _et al_ (1978) and Harsing (1982) described a high initial rate of enkephalin leakage; the latter workers quoted a loss of 25% of the tissue content over

30 min. In both cases, efflux subsequently declined to a very low, "basal" level. Patey et al (1981) found that cytoplasmic aminopeptidases appeared in the medium in which freshly chopped rat striatum was incubated, but that enzyme leakage was negligible after 30 min. incubation. In the present experiment, the cytoplasmic marker enzyme, lactate dehydrogenase (LDH) was monitored in the superfusate. The rate of LDH leakage was fairly uniform over 1 h. (table 4.5.1.3.) and the total quantity leaked was low compared to reported levels of brain LDH viz., 50 units/g (Johnson, 1960) or 44 units/g (Johnson and Whittaker, 1963). The tissue was immersed in buffer during cutting by the "Vibroslice", so that leaked enzyme may have been discarded; moreover, the slices were washed before beginning superfusion. An initially high rate of LDH leakage would not necessarily have been detected. It is nevertheless possible that the "Vibroslice", which has a different cutting action from that of the McIlwain tissue chopper used by other investigators, may have produced slices with different properties (Garthwaite et al, 1979; 1980; McIlwain, 1975). Table 4.5.1.2. shows that the striatal slices were capable of retaining an internal potassium content higher than the surrounding medium; the ability to do so argues that a high proportion of the plasma membranes of the cells in the slices remained intact, and also that the cells were performing metabolic work during the 1 h. incubation. The absolute potassium content was rather lower than that typical of fresh brain tissue; McIlwain (1975) quotes the K^+ content of the cerebral cortex of rabbit and of guinea pig as 103 and 112 μmol/g wet tissue weight, respectively. However, the process of slice preparation generally depletes tissue potassium to some extent and Cummins and McIlwain (1961) regarded a potassium content of 60-65 μmol/g wet weight as indicative of a "healthy" metabolic state in superfused slices of guinea pig cortex.

In summary, it seems probable that the loss of enkephalins, together with other cytoplasmic constituents, from damaged cells probably accounts for a major part of the total amount of ME and LE which disappeared from the tissue. Neither Osborne _et al_ (1978), Iversen _et al_ (1978) nor Harsing _et al_ (1982) could account for all the lost enkephalins by assaying the superfusate, perhaps because the activity of enkephalin degrading enzymes was not completely inhibited in these experiments. Appropriate enzyme inhibitors permit the quantitative recovery of enkephalin released by the depolarization of brain slices (De la Baume _et al_ 1982, 1983). However, the possibility remains that a proportion of the enkephalin "lost" is broken down within the slices; the experiments using bestatin and/or SQ 24,994 with striatal slices _in vitro_ were designed to test the possibility that such intracellular enkephalin-degrading enzyme activity might be susceptible to inhibition by these compounds.

5.3.2. EFFECT OF ENZYME INHIBITORS ON THE ENKEPHALIN CONTENT OF STRIATAL SLICES.

Neither 100 μM bestatin, nor 10 μM SQ 24,994, nor both drugs used together, affected the ME or LE content of striatal slices after 1 h. superfusion; neither was the ratio of ME to LE affected. (table 4.5.2.1.)

It has already been demonstrated, how such drugs protect exogenous radio-labelled LE from degradation by striatal slices or membranes. (sections 5.2.2., 5.2.3.). Inhibitors of the YGG-forming endopeptidase, enkephalinase, such as Phe-Ala (Patey et al, 1981; Harsing et al, 1982) or thiorphan (Patey et al, ;981; De la Baume et al, 1982, 1983) significantly enhance the recovery of endogenous enkephalin released from striatal slices, as will the aminopeptidase inhibitor, bestatin, while a combination of bestatin and thiorphan affords virtually complete protection of released enkephalins, (De la Baume et al, 1982, 1983). It seems reasonable to assume that the high drug concentrations used here would completely inhibit extracellular enzymes converting enkephalins to Y, YGG or YGGF. It is improbable that these extracellular enzymes metabolise intracellular enkephalins. Harsing et al (1982) claimed that 100 μM Phe-Ala could increase the enkephalin content of superfused striatal slices. Since extracellular enkephalins should have been rapidly removed from the tissue by the flow of superfusion fluid, the most likely explanation of the results of Harsing et al was that 100 μM Phe-Ala inhibited an enzyme which had been degrading enkephalins within the tissue.

There are several possible explanations for the failure of these drugs to affect intracellular enkephalin content. The findings of Harsing et al require that the striatal slices be capable of de novo enkephalin synthesis; in the absence of inhibition of the Phe Ala-sensitive catabolizing enzyme, the rate of synthesis must be supposed

to be exceeded by the rate of breakdown. It is conceivable that, in the *in vitro* system investigated here, the pool of enkephalins is neither added to by synthesis nor subtracted from by enzymic degradation, at a rate sufficient for inhibition of intracellular enzymes to have any significant effect on enkephalin content. Indeed, the loss of stored enkephalin was much more rapid from the preparation of Harsing *et al*, amounting to 67% over 75 min. superfusion. It would be necessary to account for these differences in behaviour by differences in experimental procedure. The striatal slice preparation used in these experiments was capable of metabolic work, in as much as the tissues maintained a high internal potassium content against a concentration gradient (table 4.5.1.2.). Possibly, inappropriate enzyme inhibitors were tested; however, 100 μM Phe-Ala was found not to influence enkephalin content in the *in vitro* system described here (I. Berzetei, unpublished results). A further possibility is that the compounds used in the present experiments were denied access to susceptible enzymes by their inability to cross cellular membranes. No information is available concerning bestatin; it is, however, known that SQ 24,994 can cross the blood-brain barrier (Dr. P.M. Caesar, unpublished observation).

It is noteworthy that Patey *et al* (1981) observed no effect of Phe-Ala on the enkephalin content of striatal slices. Moreover, the detailed examination of the enkephalin content of slices *in vitro* undertaken by De la Baume *et al* (1982, 1983) failed to demonstrate any effect of either thiorphan, or bestatin, or both on intracellular enkephalins.

It is difficult to believe that the rapid intracellular turnover of enkephalins, implied by the results of Harsing *et al*, is a normal physiological process, since it would necessitate a high rate of synthesis of protein precursors. The corpus striatum contains a large number of opioid perikarya, sites of precursor synthesis; in

the intact animal, enkephalin-like immunoreactive material is probably transported from these cell-bodies, down axons many of which terminate in the globus pallidus (Cuello, 1983). It may be that some change in sub-cellular compartmentation in the preparation of Harsing *et al* allowed a Phe-Ala sensitive enzyme access to stored enkephalins, and processing enzymes access to precursors. It is however intriguing that Harsing *et al*, in their succeeding publication (Zhang *et al*, 1982) did not consider the intracellular accumulation of enkephalins as an explanation of the increased striatal enkephalin levels following administration of thiorphan to mice *in vivo*.

5.4. *IN VIVO* ADMINISTRATION OF SQ 24,994.

Half an hour after *i.c.v.* injection, ME and LE in the brains of animals receiving SQ 24,994 were elevated significantly above control levels (table 4.6.).

The amounts of ME and LE in the brains of control animals were similar to those reported by Miller *et al* (1978), who found 880 pmol/g ME and 210 pmol/g LE, although the ratio of ME to LE (table 4.6) was lower than that found by Miller *et al*; Hughes *et al* (1977) also reported a higher ME/LE ratio, of about 3.5, although the quantities of enkephalins were lower by several fold (170 pmol/g ME and 46 pmol/g LE).

The obvious explanation is that SQ 24,994 protected the enkephalins from enzymic degradation. The unchanged ME to LE ratio implies that both peptides were protected to a similar degree. The methods of enkephalin purification and measurement were the same as those used in the experiments using enzyme inhibitors with striatal slices, so the effect of SQ 24,994 in this experiment is unlikely to result from an improvement in the recovery of enkephalins following removal of the brain from the animal (Methods, section 3.15).

SQ 24,994 therefore exerted its effect before the homogenization of the brain. It has already been demonstrated that SQ 24,994 is not capable of increasing intracellular ME or LE; this observation perhaps favours an extracellular location for the protected pool of enkephalin *in vivo*. It was argued above, that a rapid intracellular breakdown of enkephalins would be extremely inefficient metabolically, requiring the energy-intensive synthesis of large protein precursors. It seems likely that enkephalins are sequestered from peptidases by intra-cellular compartmentation, in view of the abundantly documented existence of intracellular enkephalin-degrading aminopeptidases (section 1.3.1.). The consensus view is that under normal physiological circumstances enkephalins in extracellular space are rapidly

broken down. Inhibition of an enzyme contributing to enkephalin inactivation might result in an accumulation of the intact peptides. Such a pool of ME and LE in extracellular space might arise by the release of enkephalins at a physiological rate, or it might result from an unusually rapid release of enkephalins, perhaps triggered by handling and killing the animals. (It would be necessary to solve this problem before using inhibitors of enkephalin degradation to measure enkephalin turnover in vivo, as suggested by Zhang et al, 1982).

If these arguments that the increase in brain enkephalin caused by SQ 24,994 takes place extracellularly are credible, then this experiment may be taken as evidence that SQ 24,994-sensitive enzymes inactivate released enkephalins in vivo. A further piece of circumstantial evidence is the observation (P.M. Caesar, unpublished) that this drug has an antinociceptive effect in mice. The experiments using radio-labelled LE identified the enzymes inhibited by SQ 24,994 as those forming YGGF and YGG. The evidence from the experiments using striatal membranes suggested that YGG formation involves an enzyme other than that which forms YGGF; YGG is probably formed directly from LE. It was also deduced that more YGG than YGGF was formed (section 5.2.2.). It is not possible to extrapolate directly from these experiments to the physiological situation. Gordon et al (1983) recently demonstrated that SQ 24,994 can inhibit two enzymes cleaving enkephalins to YGG, namely "enkephalinase" and angiotensin converting enzyme. The results presented here are thus consistent with the participation in vivo of either or both enzymes in enkephalin degradation. A recent report (Zhang et al, 1982) has shown an elevation of striatal enkephalin levels in mice following in vivo administration of thiorphan, which is probably more specific for "enkephalinase" than is SQ 24,994.

When exogenous LE is incubated with striatal tissues in vitro the

major product is Y (figures 4.4.1.1. a, 4.4.1.2). Moreover, the potent aminopeptidase inhibitor bestatin is, like thiorphan, capable of enhancing the recovery of endogenous enkephalins released from striatal slices *in vitro* (De la Baume *et al* 1982, 1983). The lack of effect of SQ 24,994 on aminopeptidase *in vitro* implies that this enzyme is not capable alone of completely degrading the pool of enkephalin arising when SQ 24,994 is administered *in vivo*. However, Zhang *et al* showed that bestatin potentiated the effect of thiorphan on striatal ME content *in vivo*. That both enkephalinase and aminopeptidase are involved in metabolizing extracellular enkephalins *in vivo* is confirmed by the results of Chaillet *et al* (1983) who showed that bestatin and thiorphan additively improve the recovery of labelled LE from the mouse brain after *i.c.v.* injection of the peptide into intact mice.

Zhang *et al* (1982) reported that *i.c.v.* thiorphan raised striatal ME content by 30% without affecting mid brain levels. The increase of about 40% in whole brain (minus cerebellum) ME and LE may disguise a much larger proportional increase, confined to the striatum.

The increase in ME produced by thiorphan in the experiments of Zhang *et al* persisted for 1 hour and was maximal 15 to 30 min. after injection. However, these workers observed that the "dipeptidyl carboxypeptidase" activity of the striatum was suppressed for 2 hours - some other mechanism is involved in homeostatic regulation of enkephalins. There was no increase in brain ME or LE, 10 min. after *i.c.v.* injection of SQ 24,994 (I. Berzetei and G. Zamboni); the time course of the action of SQ 24,994 in the rat seems to be slower than that of thiorphan in the mouse. SQ 24,994 increases enkephalin levels in mouse brain, 30 min. after intraperitoneal injection (P.M. Caesar, unpublished observation); the compound would hence appear to be transferred across the blood brain barrier.

5.5. EFFECTS OF SQ 24,994 ON THE MOUSE VAS DEFERENS BIOASSAY.

The inhibition of the twitch response of the electrically-stimulated mouse vas deferens bioassay preparation is a well established property of opiates and of opioid peptides (Hughes et al, 1975). Table 4.7 shows that the compound SQ 24,994 enhances the potency of LE on the tissue, presumably by increasing its concentration at the receptor. It is probable that in the absence of inhibitor, an SQ 24,994 - sensitive enzyme limits the concentration of LE at the receptor. However, in the absence of inhibitor the response to a single dose of LE is maintained for several minutes, although washing the preparation restores the twitch to its original magnitude. A simple assumption is that a steady-state exists; the enzyme creates a sharp concentration-gradient between the LE in the assay bath (the concentration of which is negligibly affected) and the LE at the receptor. The LE concentration in the organ bath is 2 to 3 orders of magnitude below the Km values reported for enkephalin-degrading enzymes. Under these conditions, reaction kinetics would probably be first-order, i.e., the rate of substrate breakdown would be proportional to its concentration (constant half-life). The dose-response curves in the presence and absence of inhibitor would then be approximately parallel (fig. 4.7. c).

The identity of the SQ 24,994 - sensitive enzyme is unknown. SQ 24,994 has been shown to inhibit both angiotensin converting enzyme and "enkephalinase" (Gordon et al, 1983), and in the present study inhibited both Tyr Gly Gly and Tyr Gly Gly Phe formation by brain enzymes degrading LE (section 4.1.1.). Llorens and Schwartz (1981) detected Tyr Gly Gly forming activity, attributed to enkephalinase, in rat vas deferens. McKnight et al (1983) have recently developed a "cocktail" of enzyme inhibitors (thiorphan, captopril, bestatin and Leu Leu) which enhance the potency of enkephalins on the mouse vas bioassay; their results imply that more

than one enzyme species is involved. It is highly likely that enkephalinase is a major enkephalin-degrading enzyme in the vas, while the efficacy of bestatin (McKnight et al, 1983) suggests that aminopeptidase activity is also of importance; if this is the case, it appears possible that the enkephalin-inactivating system in this peripheral tissue might be closely similar to that in the brain.

5.6. GENERAL DISCUSSION

Schwartz et al. (1980) declared that "enkephalinase" was the "acetylcholinesterase of enkephalins". McIlwain and Bachelard (1971) calculated that, if 1% of the tissue content of acetylcholine were released at a synapse, its hydrolysis would take 0.7 msec. Hudgin et al. (1981) determined the Vmax values of the "enkephalinase" and aminopeptidase activities of a striatal membrane preparation to be 170 and 592 pmol/mg/min respectively, following the method of calculation of McIlwain and Bachelard, the hydrolysis of 1% of the ME and LE content of the carpus striatum would take 6 msec. The corresponding time required for the re-uptake of noradrenaline is a few msec (Iversen, 1971). It is difficult to interpret these figures, since nothing is known of the local concentrations following the synaptic release of enkephalins; indeed, the proportion of the tissue stores available for release *in vivo* is unknown, although it is probably lower than that released following *in vitro* depolarization, which is a few percent. Information is also lacking, concerning the microscopic distribution of enkephalin-inactivating 'enzymes' in relation to sites of enkephalin release - thus the Vmax figures, measured per unit tissue weight *in vitro*, may underestimate potential rates *in vivo*. It does at least appear possible that under suitable conditions, the 'enkephalinase' and aminopeptidases in the brain have the potential to inactivate enkephalins with a rapidity which would be consistent with a function for LE and ME as neurotransmitters.

The hypothesis of de la Baume et al., that both aminopeptidase(s) and the endopeptidase 'enkephalinase' participate in the physiological inactivation of enkephalins, is consistent with the results of numerous studies reported in the literature. It is still not clear, however, to what extent either enzyme is responsible for enkephalin degradation *in vivo*. That this problem remains can be attributed only to a small extent

to a lack of specificity in the enzyme inhibitors which have generally been the diagnostic tools in this field. In fact, current experimental techniques are probably incapable of determining the amount of synaptically released enkephalin degraded in vivo by each pathway, owing to the great difficulty of purifying and quantifying the minute quantities of released enkephalins and their catabolites, while it is impossible to incorporate radio-labelled amino acids into endogenous enkephalin with any specificity. Thus only indirect approaches are presently available. Work on the purified enzymes (sections 1.3 and 1.5) has led to the development of enzyme inhibitors of some specificity. The experimental work described in Chapter 3 and 4 above initially concentrated on determining the effects of the best enzyme inhibitors available, on all the possible pathways of enkephalin degradation, using striatal membrane or slice preparations - model systems which can be regarded as being intermediate in complexity between the purified enzymes and the intact animal. It was found, in agreement with the results of many other workers, that in addition to 'enkephalinase' activity, the aminopeptidase activity present in both slices and membrane will rapidly inactivate enkephalins. It is known, moreover, that aminopeptidase activity is uniformly distributed throughout the brain (section 1.3.1). This aminopeptidase activity was inhibited both by puromycin and by bestatin, the latter being more potent. Either SQ24994 or thiorphan reduced the breakdown of enkephalins via pathways leading to Tyr-Gly-Gly-Phe or Tyr-Gly-Gly, but in the presence of either drug, aminopeptidase activity was still capable of degrading more than 70% of the LE substrate. Nevertheless, SQ24994 was able to induce analgesia in an animal model (Dr P. M. Caesar, unpublished observation)

and significantly to increase the ME and LE content of rat brain *in vivo* (section 4.6). It seems paradoxical that a considerable pool of enkephalins could thus escape hydrolysis by aminopeptidases, probably for tens of minutes; it is argued in sections 5.3 and 5.4 that this pool of enkephalins is unlikely to have been intracellular. Even if it is granted that perhaps only a small subset of aminopeptidases are strategically localized for the synaptic degradation of enkephalins, so that available measurements of total aminopeptidase activity in slices and membranes are probably over-estimates of the physiologically relevant aminopeptidase activity, it is hard to imagine that the accumulated peptide did not diffuse away from the sites of its release, and encounter a high level of aminopeptidase activity. Bearing in mind the different distributions of the two classes of enzyme, one might postulate a scheme in which 'enkephalinase' was mainly responsible for lowering the synaptic concentration of enkephalins while aminopeptidase could inactivate enkephalins which might have diffused away from the site of release. It is interesting that Zhang *et al*. (1982) reported that bestatin further increases the raised ME content of the striatum, which is found when thiorphan is given *in vivo*. However, the efficacy of SQ24994 or thiorphan given alone, either as antinociceptives or in elevating endogenous enkephalin levels, suggests that, at least in the animal model studied here, the activity of aminopeptidases alone is not sufficient to inactivate released enkephalins. Further *in vivo* experimentation seems necessary to clarify this situation; unfortunately, as remarked above, only indirect approaches are available. Although the demonstration of nalaxane-reversible antinociceptive effects has been of great value in implicating endogenous opioids in physiological

processes, the investigation of alternative behavioural models using, for example, pupillary effects, would be welcome. It is conceivable, for instance, that more selective effects of enzyme inhibitors would then become apparent, if there were differences in the mode of inactivation of enkephalins between different physiological systems.

Radio-labelled enkephalins can readily be introduced into the CNS; Chaillet et al. (1983) showed that both bestatin and thiorphan could improve the recovery of exogenous LE from mouse brain in vivo, and that their effects were additive. More detailed experiments appear to be justified, in which all the possible LE catabolites could be quantified; it would also be possible to test nociception in each animal immediately before sampling. As an alternative to enzyme inhibitors, chemically modified enkephalin analogues may be administered in behavioural experiments. Some results of such studies have been described in section 1.6. To allow differences in antinociceptive potency to be attributed to differences in resistance to enzymic attack it is necessary to take into account possible differences in the affinity of these analogues for apioid receptors. Modified peptides have to be artificially introduced into the CNS, raising problems of interpretation common also to studies using labelled enkephalins - exogenous substrate may meet a different population of peptidases from that encountered by the endogenously released transmitter. It is possible that differences exist in the mechanisms of inactivation of enkephalins in different regions of the brain; such regional variations might not be resolved by the techniques now available. Moving to the other end of the scale of integration, it may be possible to design electrophysiological experiments in which

specific peptidase inhibitors would be used to modify the post-synaptic effects of synaptically-released endogenous enkephalin. At the moment, the 'simplest' systems in which a response to opioid administration can be demonstrated are *in vitro* bioassay preparations such as the mouse vas deferens utilized in the present study. It was found that SQ24994 enhanced the sensitivity of the bioassay to LE, which implies that an SQ24994-sensitive enzyme regulates the concentration of exogenous LE at these peripheral receptors, although the LE concentration in the organ-bath was far lower than the Km values quoted for either enkephalinase or aminopeptidase. LE concentrations applied to the tissue ranged from 2 nM to 40 nM, while the dissociation constants of opiod receptors lie in the low nM range (Chang *et al.*, 1979), so that the higher LE concentrations elicited almost a maximal physiological response. While the relevance of this peripheral tissue model to the brain is uncertain, it was valuable in demonstrating the effectiveness in enkephalin inactivation of enzymes working at substrate concentrations well below their Km's. The compound SQ24994 inhibits Tyr-Gly-Gly-formation rather than amino-peptidase activity (Table 4.4.1.1c) but the results of McKnight *et al.* (1983) suggest that aminopeptidases are also active in enkephalin breakdown in this tissue.

On balance, it seems unlikely that aminopeptidase(s) and the neutral endopeptidase 'enkephalinase' have equivalent functions in the breakdown of enkephalins *in vivo*; perhaps their activities are in some ways complementary, in view of their different specificities and distributions. It may even be that the inactivating system varies locally and that this variation is of functional significance, regulating the extent and

duration of action of the enkephalinergic signal. Enkephalins often co-exist with "classical" transmitters, for example with noradrenalin in sympathetic neurones, and with adrenalin and noradrenalin in the adrenal medulla (Schultzberg et al., 1987; Wilson et al., 1980), and in catecholaminergic neurones of the cat locus caeruleus (Charnay et al., 1982); as pointed out by Lundberg and Hökfelt (1983), where peptide and "classical" transmitter are released together, the peptide is often responsible for a component of the response which is slower in onset, and persists longer, than that of, for example, acetylcholine or noradrenalin - although peptidergic actions need not necessarily be slower than those of the "classical" transmitters (Iversen, 1983). Contacts between enkephalinergic neurones and their presumed target cells are not invariably tight synaptic junctions, so that the released enkephalins may have to diffuse some distance to interact with opioid receptors. Cuello (1983) proposed non-synaptic interactions between enkephalinergic axons and dendrites in the caudate putamen and the terminals of nigrastriatal dopaminergic neurones, to which a proportion of striatal enkephalinase has been localized (Llorens-Cortes et al., 1979). On the other hand, the very susceptibility of enkephalins to enzymic degradation argues that they may be particularly associated with rapidly-terminated signalling involving opioid receptors. The recognition of such a small peptide by peptidases must depend on its primary structure, whereas a larger peptide, for example, β-endorphin, can possess a tertiary structure conferring resistance to peptidases (Austen and Smythe, 1977). It is interesting that, unlike enkephalins, many neuropeptides have blocked N- or C-termini, e.g., oxytocin, vasopressin and neurotensin. The inactivation of such peptides presumably involves endopeptidases, or possibly very specialized exopeptidases. It is likely that certain features of the

sequences of neuropeptides control their susceptibility to attack by endopeptidases; there is a high incidence of proline in bradykinin, substance P and neurotensin, and oxytocin and vasopressin contain intra-chain disulphide bridges. The number of peptides which may be neuro-transmitters or neuromodulators far exceeds the number of putative neuro-peptidases (Iversen, 1983; Beaumont, 1983). Perhaps each peptidase inactivates several different peptides in vivo, since peptidases generally exhibit broad specificities in vitro, and the ranges of peptide substrates attacked by different peptidases seem to overlap; for example, several enzymic species of aminopeptidases can attack enkephalins; angiotensin converting enzyme and "enkephalinase" have the same sequence specificity in their cleavage, both of bradykinin and of enkephalin; and substance P can be hydrolysed either by "enkephalinase" (Matsas et al., 1983) or by the "substance P degrading enzyme" (Lee et al., 1981) - and also by other peptidases. Such interactions between different peptidergic systems at the level of the inactivation mechanisms are potential pitfalls in the interpretation of the effects observed when specific peptidase inhibitors are administered to intact animals. The preceding discussion has concentrated on the properties of aminopeptidases "enkephalinase", and other enzymes were detected in the in vitro experimental work. An enzyme activity is present in both striatal slice and membrane preparations, which generates Tyr-Gly from LE (section 5.2.2); this activity may be the "enkephalinase B" of Gorenstein and Snyder (1979, 1980). The results presented in sections 5.2.2 and 5.2.3 concur with the generally accepted view that this dipeptidyl-aminopeptidase-like activity is not of major importance in enkephalin breakdown. A carboxypeptidase activity,

probably of similar specificity to carboxypeptidase A, was also detected on both striatal slices and membranes (sections 5.2.2, 5.2.3) and hydrolyzed exogenous LE at a rate approaching that at which Tyr-Gly-Gly was formed. The participation of an enzyme of this class has not previously been reported; although probably a minor pathway of enkephalin breakdown, its importance deserves to be investigated. It is possible that the enzyme is involved in the cleavage of other neuropeptides.

We are a very long way from understanding the complexities of peptide inactivation engendered by the partial selectivities of the peptidases. However, clear-cut differences in specificity may be a valuable indication that particular enzymes and peptides are involved in functionally discrete systems.

The functions of neutral endopeptidase in the peripheral organs are unknown but are certainly not confined to enkephalin inactivation. In many tissues the endopeptidase has functions unrelated to the nervous system. Danielsen _et al_. (1980) have presented evidence that large quantities of neutral endopeptidase activity, enzymically, molecularly and immunologically similar to the kidney microvillar neutral endopeptidase E.C. 3.4.24.11 exists on the membranes of the intestinal microvilli, lining the lumen; presumably the enzyme participates in digestion. In section 1.5.2 above, evidence was reviewed which supported the conclusion that the brain neutral endopeptidase "enkephalinase" is very similar if not identical to the analogous kidney enzyme (Matsas et al., 1983). Recently Almenoff and Orlowski (1984) demonstrated that the brain enzyme had a very strong cross-reactivity against an immune serum raised against kidney endopeptidase, but that the kidney enzyme bears antigenic determinants

which the brain enzyme lacks. There were also small but significant differences in the Ki's of the enzymes for phosphoramidon and for two novel inhibitors of the carboxyalkyl type (Almenoff and Orlowski, 1983). These discrepancies may be attributable fo differences in glycosylation rather than amino acid sequence, and no differences in substrate specificity were detected by Almenoff and Orlowski. At the moment it seems that these peptidases are not absolutely identical but their very close similarity is certain.

The occurrence of this neuropeptidase in large amounts in visceral organs has facilitated the development of specific antisera. A monoclonal antibody raised by Gee et al. (1983) against the kidney neutral endopeptidase was effective in the purification by immunoaffinity chromatography of the enzymes from kidney, intestine and brain. In the future such sera may permit the immunohistochemical mapping of the endopeptidase in brain tissue.

Not only the neutral endopeptidase, but many other putative neuropeptidases are Zn-enzymes and the successes in the rational synthesis of specific, active-site-directed inhibitors for "enkephalinase", carboxypeptidases A and B and angiotensin-converting enzyme should lead to the future development of a spectrum of specific inhibitors against novel peptidases.

Further investigation of the distribution and function of neuropeptidases, both as inactivating and processing enzymes, will surely illuminate our understanding of neuronal communication.

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