BIOLOGICAL BARRIERS TO THE NASAL DELIVERY OF PEPTIDE DRUGS

by

PAULA A. HOLBROOK

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SUMMARY

The nasal absorption of larger peptide and protein drugs is generally low. The importance of the mucus layer and enzymic degradation in reducing absorption were investigated.

Reversed-phase high-performance liquid chromatographic (HPLC) methods were developed to assay a variety of compounds.

Pig gastric mucus (PGM) was selected to investigate the importance of the mucus layer. A method of treating and storing PGM was developed and evaluated which was representative of the gel *in vivo*.

The nature of the mucus barrier was evaluated *in vitro* with three-compartment diffusion cells and a series of compounds with differing physicochemical properties. Mucus retarded the diffusion of all the compounds with molecular weight and charge exerting a marked effect. Binding to mucus was investigated by a centrifugation method. All of the compounds tested were found to bind to mucus with the exception of the negatively charged molecule benzoic acid. The small peptides did not demonstrate greater binding to mucus than any of the other compounds evaluated.

The effect of some absorption enhancers upon the rate of diffusion of tryptophan through mucus was determined *in vitro*. At the concentrations employed the enhancers EDTA, N-acetylcysteine and taurodeoxycholic acid exerted no effect, whilst taurocholic acid and cholic acid, were found to slightly reduce the rate of diffusion.

The intracellular and luminal proteolytic activity of the nose was investigated in the sheep animal model with a nasal mucosal homogenate and a nasal wash preparation respectively and a series of chemically similar peptides. Hydrolysis was also investigated with the proteolytic enzymes carboxypeptidase A, cytosolic leucine aminopeptidase and microsomal leucine aminopeptidase.

Sheep nasal mucosa possesses significant peptide hydrolase activity capable of degrading all the substrates tested. Considerable variation in susceptibility was observed. Degradation occurred exclusively at the peptide bond between the aromatic amino acid and glycine, indicating some specificity for aromatic amino acids. Hydrolysis profiles indicated the presence of both aminopeptidase and carboxypeptidase enzymes. The specific activity of the microsomal fraction was found to be greater than the cytosolic fraction. Hydrolysis in the nasal wash indicated the presence of either luminal or loosely-bound proteases, which can degrade peptide substrates. The same specificity for aromatic amino acids was observed and aminopeptidase activity demonstrated. The specific activity of the nasal wash was smaller than that of the homogenate.

KEYWORDS: Intranasal; peptide; mucus; absorption enhancers; proteolytic enzymes.

To Elsie Minikin

(1900-1989)

"There is no such word as can't"

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ABBREVIATIONS

AT	atenolol
AUFS	absorbance units full scale
BA	benzoic acid
BC	benzalkonium chloride
BSA	bovine serum albumin
CA	cholic acid (sodium salt)
CBF	cilia beat frequency
CEN	centrifugation
CPA	carboxypeptidase A
cm	centimetres
DDAVP	desmopressin
DEA	diethylamine
DEAE	diethylaminoethyl
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DPM	disintegrations per minute
DSM	degradable starch microspheres
DW	distilled water
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
F	phenylalanine
FF	phenylalanylphenylalanine
FFF	phenylalanylphenylalanylphenylalanine
FG	phenylalanylglycine
FGG	phenylalanylglycylglycine
FGGF	phenylalanylglycylglycylphenylalanine
FL	phenylalanylleucine
G	glycine
G	elasticity
G'	storage (elastic) modulus
G''	loss (viscous) modulus
GF	glycylphenylalanine
GFL	glycylphenylalanylleucine

GG	glycylglycine
GGF	glycylglycylphenylalanine
GGFL	Des-tyrosine leucine enkephalin acetate salt
GGG	glycylglycylglycine
g	grammes
g	gravity
GH	growth hormone
GHRH	growth hormone-releasing-hormone
GI	gastrointestinal
GP's	glycoprotein molecules
HPLC	high performance liquid chromatography
h	hours
I.S.	internal standard
J	flux (rate of transport per unit area of surface)
Kp	permeability constant
Km	Michaelis constant
L	leucine
L	litre
LAP-C	cytosolic leucine aminopeptidase
LAP-M	microsomal leucine aminopeptidase
LHRH	luteinising hormone-releasing-hormone
ln	natural logarithm (to base 2)
log	logarithm (to base 10)
ME	L-phenylalanine methyl ester
MeCN	methylcyanide (acetonitrile)
mg	milligrammes
min	minutes
mL	millilitres
mM	millimolar
mmol	millimoles
MP	methylparaben
NAC	N-acetylcysteine
NGP's	nasal glycoprotein molecules
NM	native mucus
NP	N-acetyl-L-phenylalanine
nm	nanometres

ODS	octadecylsilane
OX	oxprenolol
P	partition coefficient
PGG	purified glycoprotein gel
PHE	L-phenylalanine
PMSF	phenylmethylsulphonylfluoride
PR	DL-propranolol
pKa	$\log_{10}$ dissociation constant
r	correlation coefficient
rpm	revolutions per minute
s	seconds
SA	salicylic acid
sem	standard error of the mean
STDC	sodium taurodeoxycholate
STDHF	sodium taurodihydrofusidate
TA	taurocholic acid (sodium salt)
TDA	taurodeoxycholic acid (sodium salt)
TFA	triflluoroacetic acid
TH	thiomersal
T_L	lag time
TRP	L-tryptophan
UF	ultrafiltration
UV	ultra-violet
v/v	volume in volume
Vmax	maximum velocity of an enzyme-catalysed reaction.
W	tryptophan
WG	trptophylglycine
WGG	trptophylglycylglycine
W/O	water in oil
w/v	weight in volume (g/100 mL)
Y	tyrosine
YGGFL	leucine enkephalin acetate salt
[3H] TRP	tritiated tryptophan
η	viscosity
γ	strain
µg	micrograms

µL	microlitres
µM	micromolar
µmol	micromoles
τ	applied stress
°C	degrees celsius
%	percentage

CHAPTER 1
INTRODUCTION

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INTRODUCTION

1.1 BACKGROUND

Peptide molecules are compounds which contain two or more amino acids, linked together by amide bonds between the α-carboxyl group and α-amino group of the adjacent residues. The molecules are named in accordance with the number of residues they contain. Hence, a dipeptide contains two amino acids, a tripeptide three, a tetrapeptide four and a polypeptide more than ten.

The larger, macromolecular structures built of amino acids are termed proteins. Although no clear demarcation exists, proteins are generally considered to have a minimum chain length of about 50 amino acids, corresponding to molecular weights near 5000. The larger, more complicated proteins may contain several thousand residues and consequently, have molecular weights of several million (Mahler and Cordes, 1971).

Protein molecules are essential components of all living organisms and serve a variety of diverse functions. In man, proteins are the principal components of cell protoplasm and also serve as enzymes, antibodies, structural elements, transport devices and hormones (Siddiqui and Chien, 1987; Mahler and Cordes, 1971). These compounds have become of great interest to the pharmaceutical industry as knowledge of their biological function has increased and biotechnology has provided the ability to replicate proteins and peptides in large quantities (Elander, 1985; Dibner, 1986; Lee, 1986b; Sadee, 1986; Baum, 1987). The role that they will play in medicinal use is, however, subject to some debate.

Firstly, they may be viewed as tools to probe the nature and mechanism of particular disease states, which may subsequently lead to the synthesis of more traditional organic molecules. This approach has led to the development of orally effective, long-acting 3-substituted benzodiazepines, which are selective antagonists at cholecystokinin CCK-A and CCK-B receptors following from the receptor-based screening

of asperlicin (Bock, 1991). A second approach has been to locate the active part of the molecule and use a smaller peptide or peptide analogue for therapeutic use (Veber et al., 1990). One example of this methodology is the compound octreotide acetate (trade name Sandostatin), which is a fragment of the somatostatin molecule substituted with a D-amino acid at one position to improve stability (O'Dorisio et al., 1991). The alternative approach is to use the whole intact molecule produced by biotechnology, such as in insulin therapy for diabetic patients, or to chemically modify the peptide. The chemical modifications pursued include the production of prodrugs (Bundgaard and Moss, 1989; Kahns and Bundgaard, 1991; Moss and Bundgaard, 1991; Rasmussen and Bundgaard, 1991), the substitution of alternative amino acids, particularly D-amino acids in the molecule (Best et al., 1990) and cyclisation (Hruby, 1985).

The use of peptide and proteins in therapy is obviously not a new phenomena. The protein hormone insulin, purified from pigs and cattle, has been used to treat diabetes since its discovery in 1921 (Chien and Banga, 1989). What is new, and has huge potential for the future, is the ability of biotechnology to replicate exactly, and in large quantities peptides of human origin. Hence, it is now possible for diabetics to be administered human insulin, with reduced immunological, allergic and local reactions (Brogden and Heel, 1987).

The therapeutic possibilities are not merely restricted to diabetics and peptides are becoming one of the fastest growth areas in the search for new drugs. For instance, peptides are currently being evaluated for the treatment of viral diseases, cancer, anovulation, dwarfism, pain, infertility, wounds and burns, blood clots, immune disorders and birth control (Lee, 1986a; Su, 1986; Banga and Chien, 1988). Some examples are given in table 1.

Table 1.1 Some examples of therapeutic peptides and their application (Lee, 1986a; Su, 1986; Banga and Chien, 1988).

peptide	function/application
cardiovascular-active	
angiotensin II antagonist	lowering blood pressure
atriopeptin	regulating cardiovascular function and electrolyte and fluid balance
bradykinin	improving peripheral circulation
calcitonin gene-related factor	vasodilator
tissue plasminogen factor	dissolution of blood clots
CNS-active	
cholecystokinin (CCK-8 and CCK-32)	suppressing appetite
delta sleep-inducing hormone	improvement of disturbed sleep
γ-endorphin	pain relief
melanocyte inhibiting factor-1	mood improvements in depressed patients
melanocyte stimulating hormone	improving attention span
GI-active	
gastrin antagonist	reducing gastric acid secretion
neurotension	inhibiting secretion of gastric
somatostatin	reduced bleeding of gastric ulcers
Immunomodulating	
enkephalins	stimulating lymphocyte blasto-genesis
interferon	enhancing killer cell activity
tumour necrosis factor	control of polymorphonuclear functions
Metabolism-modulating	
human growth hormone	treating hypopituitary dwarfism
gonadotrophins	stimulating ovulation,
insulin	treating diabetes mellitus
vasopressin	treating diabetes insipidus
thyrotropin-releasing hormone	prolonging infertility and lactation in breast feeding women
oxytocin	maintaining labour

Clinical usage of these molecules has, however, been limited. This has been due, predominantly, to instability in the gastrointestinal tract, to poor transport across the mucosal membrane and to extensive first pass elimination (Humphrey, 1986). These factors have restricted delivery to the parenteral routes which, although acceptable for short term or life-threatening disorders, are unsuitable for chronic administration due to patient compliance.

The methods being pursued to circumvent these problems are varied and include improving parenteral and oral delivery (Davis, 1986), in addition to exploring the feasibility of alternative routes of administration, such as the nasal (Su et al., 1985), pulmonary (O'Hagan and Illum, 1990), buccal (Merkle et al., 1986), rectal (Van Hoogdalem et al., 1989a and b; 1990), vaginal (Okada et al., 1982), ocular (Chiou and Chuang, 1988), and transdermal routes (Banerjee and Ritschel, 1989a and b).

Methods to improve parenteral delivery have largely been developed for insulin and have been reviewed by Chien and Banga (1989). One of the lines of research is to reduce the frequency of injections by the use of long acting injectables, implants and colloidal preparations. One commercially available peptide, Zoladex (trade name), is delivered as an injectable implant, which gives sustained release of the decapeptide for one month. After this time a further injection is required, although work on a trimonthly preparation is currently underway (Fildes, 1991; Furr and Hutchinson, 1991).

Ways of improving oral delivery have followed two main lines of investigation. The first is based on the synthesis of metabolism-resistant analogues (Hruby, 1985), which has had some success in producing more stable analogues, but has not improved mucosal transport or reduced the rapid clearance of the compounds by the liver (Humphrey and Ringrose, 1986). Additionally, the modifications have been observed to have a marked effect upon the potencies of the compounds (Hruby, 1985).

Improved peptide formulation has been the other line of investigation, where the formulation acts as a carrier or protects the peptide from degradation. The formulation approach has been studied extensively with respect to insulin. The formulations tested include water-in-oil-in-water emulsions (Shichiri et al., 1975), liposomes (Patel et al., 1978; 1982), nanoparticles (Oppenheim et al., 1982; Damge et al., 1988) and soft gelatin capsules with different coatings (Touitou et al., 1986). None of these as yet have improved oral absorption sufficiently to be of therapeutic benefit (Lee, 1990). Coadministration of protease inhibitors with insulin has also been investigated (Fujii et al., 1985). This line of research is expected to be more useful against luminal than brush border or intracellular proteases and means that coadministration may have a limited success depending upon the type of protease and its subcellular location. If the principal proteolytic activity is cytosolic, it is imperative that the protease inhibitors are absorbed into the cell. This may be difficult in view of the fact that protease inhibitors are often too large or hydrophilic to traverse biological membranes (Lee, 1990).

The alternative delivery routes have been implicated as useful for peptide administration for several reasons. The avoidance of first pass elimination, reduced enzymic activity in comparison with the oral route, the possibility of increased membrane permeability promoting paracellular absorption (Hayashi et al., 1985) and reduced dilution effects (increasing the concentration gradient for absorption) all suggested that absorption from these routes would be higher than from the GI tract. In reality, absorption from these routes to date has been disappointing and indicates the complexity of the barriers to peptide delivery. This thesis will focus on the nasal delivery of peptide molecules.

1.2 NASAL DRUG DELIVERY.

1.2.1 Nasal absorption of drug molecules

The nose has been used as a route of drug delivery since ancient times. In India, Nasaya Karma (nasal therapy), was a recognised form of treatment by the Hindu Ayurdevic medicine system and in South

America and Africa plant snuffs, containing psychotropic hallucinogenic compounds, were used by witch doctors for disease treatment (Chien and Chang, 1985).

The nasal route of delivery has several features which render it suitable for drug administration. These include easy administration for the patient, a large surface area for absorption (provided by the folds of the turbinates), a highly vascularised bed providing a good blood supply, rapid absorption, avoidance of first pass elimination and postulated reduced enzymic activity (Harris, 1986; Longenecker, 1986).

Intranasal drug delivery has however, largely been confined to drugs which produce local effects in the nose including steroids, sodium cromoglycate, antihistamines and decongestants for treatment of nasal diseases such as rhinitis, hay fever, nasal polyps and secondary nasal oedema associated with sinusitis and the common cold (Dushay and Johnson, 1989; Lawrence and Patterson, 1990). The observation that nasal administration could produce systemic side effects in some cases (Parr, 1983) suggested that nasal delivery could be a viable means of systemic delivery for compounds which could not successfully be administered orally.

The nasal absorption of several compounds which exhibit either poor absorption from the GI tract or undergo extensive first pass elimination, have been evaluated in animals and humans, as a means of improving bioavailability. These have been extensively reviewed by Chien et al. (1989) and will only briefly be discussed here. Sulbencillin and two cephalosporins, cephacetrile and cefazolin, are poorly absorbed from the GI tract, which is ascribed to their high water solubility and lack of lipophilic properties. The three compounds were administered orally, intranasally and intramuscularly to rats *in vivo* and urinary recoveries determined as an indication of absorption. Poor oral absorption was observed for each compound. The urinary recoveries after nasal administration were 50% of the recovered concentration after intramuscular administration indicating

that significant nasal absorption had occurred (Hirai et al., 1981a). Vitamin B_{12} which is also poorly absorbed after oral administration, has been shown to produce adequate clinical and haematological response after intranasal administration to patients suffering from pernicious anaemia and is commercially available in a nasal dosage form (Monto and Rebuck, 1954; Nudelman, 1987). It should be noted that not all drugs with low oral bioavailability are well absorbed from the nasal mucosa. In the case of the aminoglycoside gentamicin, poor absorption from the nasal mucosa of rats and sheep was also observed in the absence of absorption promoters (Illum et al., 1988). The lack of absorption is believed to be a function of the molecular weight, hydrophilicity and high polarity of the drug, which limit transcellular transport across the nasal mucosa.

Sex hormones, some cardiovascular drugs and analgesics undergo extensive first pass elimination after oral delivery and have subsequently been delivered intranasally in an attempt to circumvent this problem. The nasal delivery of progestational steroids, oestradiol and the synthetic contraceptive steroid 17α-ethinyloestradiol to rats produced significantly higher bioavailabilities than the oral route in each case (Barwarshi-Nassar et al., 1989a and b; Schipper et al., 1990). The administration of the cardiovascular drug propranolol to the nasal mucosa of rats in solution and to humans in a 2% methylcellulose gel, has also been observed to be as effective as intravenous delivery (Hussain et al., 1979; 1980). A similar improvement has been reported for the cardiovascular drugs nicardipine (Visor et al., 1986) and clofilium tosylate (Su et al., 1984) and the analgesic buprenorphine (Eriksen et al., 1989). In summary, it would appear that small, lipophilic compounds, such as propranolol, which have low oral bioavailabilities due to first pass elimination, are well absorbed from the nasal cavity and possess bioavailabilities comparable to the parenteral route.

1.2.2 Nasal absorption of small peptides

Numerous classes of peptide and protein molecules have been delivered intranasally and have been reported to cross the nasal mucosa in animals and man to different degrees. Some small peptides, such as the tripeptide, thyrotropin-releasing hormone and the pentapeptide enkephalin-analogues, DADLE and metkephamid, cross the rat nasal mucosa successfully, with bioavailabilities of 20%, 59% and 102% respectively, in the absence of enhancers (Sandow and Petri, 1985; Su et al., 1985). Similarly, the hexapeptide ORG2766, a metabolically stabilised ACTH(4-9) analogue, has a bioavailability of 15 % after intranasal delivery to rats (Schipper et al., 1991a). The absorption of other small peptides is not, unfortunately, as successful. The bioavailability of the small pentapeptide leucine enkephalin (YGGFL), after nasal administration, is currently undetermined, due to the lack of sensitive analytical techniques to determine YGGFL and its metabolites in plasma, but is expected to be less than 10%, as it has been shown that this compound undergoes rapid hydrolysis in the nasal cavity and nasal perfusate of rats and humans (Hussain et al., 1985b; Faraj et al., 1990a and b; Hussain et al., 1990). It has also been observed that the disappearance of the dipeptide L-tyrosyl-L-tyrosine and its lipophilic derivative L-tyrosyl-L-tyrosine methyl ester, from the rat nasal cavity, was due mainly to enzymic hydrolysis to L-tyrosine (Hussain et al., 1985a) rather then due to absorption. Tengamnuay and Mitra (1988a) did not observe any significant absorption of the dipeptides, L-tyrosyl-glycine, L-tyrosyl-L-arginine (kyotorphin) or L-tyrosyl-D-arginine from the rat nasal mucosa even when hydrolysis was suppressed.

1.2.3 Nasal absorption of larger peptides

The bioavailability of larger peptides after delivery *via* the nose is generally 1-20%, which is proposed to depend upon the molecular weight and physicochemical properties of the peptide (Harris, 1986). Despite this, the posterior pituitary hormones, oxytocin, vasopressin and its synthetic analogues, lypressin and desmopressin (DDAVP), are commercially available in nasal dosage forms, in addition to parenteral delivery dosage forms (Chien and Chang, 1987). Oxytocin is

a decapeptide, which is used to induce labour, to assist an ongoing abortion, or to increase milk flow (Hoover, 1971). The degree of absorption from the nose has been shown to be 1-2% of the applied dose, in a study of 266 women (Hendricks and Pose, 1961). Vasopressin, lypressin and DDAVP are employed for the treatment of diabetes insipidus and comprise 9, 8 and 8 amino acid residues respectively. In humans, the greatest bioavailability of DDAVP is reported to be 6 % (Lethagen et al., 1987). The low bioavailability may be due partially to enzymic degradation, as DDAVP is rapidly hydrolysed in nasal homogenates from the albino rabbit (Critchley, 1989)

Another class of peptides of similar molecular weight are the anterior pituitary hormones, derived from luteinizing hormone-releasing-hormone (LHRH). LHRH is a decapeptide which stimulates the gonadotrophs of the anterior pituitary to secrete both luteinising hormone and follicle stimulating hormone. A series of LHRH agonists have been developed, which are either LHRH decapeptides with amino acid substitutions, or which differ from LHRH in their C-terminal sequence. The nasal bioavailability of LHRH and LHRH agonists is low, being 1-1.5% for LHRH in humans (Fink et al., 1974) and only 2% for nafarelin in rhesus monkeys (Anik et al., 1984). Buserelin, a LHRH agonist, is commercially available as a nasal spray, and exhibits a bioavailability in humans of 2.5% (Larsen et al., 1987). It is used clinically for the treatment of endometriosis (Donnez et al., 1989), androgen-dependent prostatic cancer (Tolis et al., 1982; Faure et al., 1983), breast cancer (Klijn and de Jong, 1982), precocious puberty (Luder et al., 1984) and in assisted conception cycles (Owen et al., 1989). The therapeutic effect is a result of down-regulation of LHRH receptors, which in turn suppresses gonadotropin production.

1.2.4 Nasal absorption of high molecular weight hormones

The intranasal delivery of three higher molecular weight peptide hormones, insulin, glucagon and calcitonin, have undergone extensive study. Insulin is the most widely used therapeutic peptide and has been extensively studied and reviewed, as a model compound for

evaluating peptide drug delivery methodology (Flier et al., 1985; Longenecker, 1986; Eppstein and Longenecker, 1988; Pontiroli et al., 1989b). Insulin, is a protein hormone with a molecular weight of 5808, comprising 51 amino acid residues. Physiologically it is involved in the control of blood glucose levels and is employed clinically in the maintenance of insulin-dependent diabetics (Chien and Banga, 1989). It was first delivered nasally to man in 1922, where it was reported to produce: "A weak, doubtful, or frankly negative effect" (Woodyatt, 1922). Work on nasal insulin delivery has continued since this time with limited success. The major problems encountered have been low bioavailability, unreliable dosing and acute local toxicity of formulations containing absorption enhancers (Longenecker, 1986). Numerous studies have attempted to address these problems using a range of absorption enhancers, improved intranasal formulations and alternative delivery devices.

The bioavailability of intranasal insulin has been improved by the administration of several different absorption enhancers. These include surfactants (Hirai et al., 1981b), bile salts (Hirai, 1978; Gordon et al., 1985; Aungst et al., 1988b), fusidate derivatives (Longenecker et al., 1987; Deurloo et al., 1989; Patapoff et al., 1990), medium chain fatty acids (Mishima et al., 1987), bile salt-fatty acid mixed micelles (Tengamnuay and Mitra, 1990), phospholipids (Hansen et al., 1988; Illum et al., 1989; Chandler et al., 1990) and most recently cyclodextrins (Merkus et al., 1991; Schipper et al., 1991b; Watanabe et al., 1991). The greatest enhancement was achieved in a formulation containing insulin and 5% dimethyl-γ-cyclodextrin, which produced a bioavailability of 108.9% after nasal administration to rats (Merkus et al., 1991). This was determined from both blood glucose and insulin levels. They proposed that the mechanism of action may be lipid extraction from the mucosal membrane. The administration of the same formulation to rabbits by the same group of workers, produced a negligible absorbance of insulin (Schipper et al., 1991b).

While all of the compounds listed above have increased bioavailability to different degrees, several workers have reported that a deleterious

effect is also exerted upon the nasal epithelium (Hirai et al., 1981c; Su et al., 1985; Ennis et al., 1990). The damage has been assessed by blood cell lysis and morphological evaluation using microscopic techniques. A range of effects have been observed from slight mucus discharge, to reduction of the epithelium to a thin, non-ciliated layer of cells and extensive cell necrosis. These effects have recently been reviewed by Lee et al. (1991). There is evidence that in the case of nonionic surfactants the degree of absorption is proportional to the extent of damage caused, as indicated by lysis of red blood cells (Hirai et al., 1981c). In contrast, is the proposal that for some compounds the mechanism of enhancement is independent of the membrane damaging process, as in the case of α-lysophosphatidylcholine-decanoyl and sodium taurodihydrofusidate (STDHF) (Chandler et al., 1990, Ennis et al., 1990).

An additional problem for the chronic use of absorption enhancers, has been the observation that six bile salts, (cholate, taurocholate, glycocholate, deoxycholate, taurodeoxycholate and glycodeoxycholate), used as absorption enhancers, caused ciliostasis in chicken ciliated embryonal tracheal tissue (Duchateau et al., 1986b). A similar ciliostatic effect was obtained using human adenoid tissue and the enhancing agents STDHF, deoxycholic acid, taurocholic acid, glycocholic acid and laureth-9 (Hermens et al., 1990). This is unacceptable for routine delivery of compounds to the nasal mucosa. The mucociliary clearance mechanism is the first line of defence against noxious agents for protection of the respiratory tract. Impairment of the defence mechanism may lead to respiratory infections, as a consequence of increased residence time of toxic materials and accumulation of secretions in the airways (Wolff, 1986). Abnormal mucus clearance is associated with a variety of conditions such as chronic rhinitis, sinusitis and bronchitis (Afzelius, 1979).

Any long term, harmful effects of absorption enhancers in humans is as yet unclear, but irritation has been reported in clinical trials of intranasal insulin containing bile salts (Flier et al., 1985), polyoxyethylene-9-lauryl ether (Salzman et al., 1985) and

phospholipids (Hansen et al., 1988). A nasal formulation of insulin containing 1% w/v STDHF is undergoing clinical trials in humans, which may indicate whether chronic use of an enhancing agent does lead to damage of the nasal cavity. The compound STDHF at concentrations greater than 0.3% w/v, was observed to cause irreversible ciliostasis *in vitro* (Hermens et al., 1990). At variance to this, was the observation that no loss of mucociliary clearance was observed in dogs after 90 days of thrice daily administration to dogs *in vivo* (Ennis et al., 1990).

Glucagon, a hormone with 29 amino acid residues, plays an important role in the physiological regulation of blood sugar. It is used therapeutically to correct hypoglycaemic shock emergencies, which afflict insulin-dependent diabetics. The compound is not absorbed after nasal administration to healthy human volunteers, but is absorbed if coadministered with dihydrofusinate or glycholate, as either a spray or a powder (Pontiroli et al., 1989a). This is proposed as a feasible delivery system for the rapid resolution of hypoglycaemic shock, which diabetics could quickly and easily administer themselves (Pontiroli et al., 1989b)

The human growth hormone-releasing hormones (GHRHs) stimulate growth hormone (GH) release in normal subjects and patients with GH deficiency. Human GH is currently delivered by subcutaneous or intramuscular injection to children diagnosed as GH deficient. This therapy is continued for several years. Nasal delivery of GHRH and GH has been explored as an alternative non-invasive means of administration. The intranasal administration of GHRH to men has been observed to stimulate GH release, but a 300-fold higher dose of the peptide was required to achieve a similar response to an intravenous dose (Evans et al., 1985). Other workers have concluded that the rat nasal mucosa is essentially impermeable to therapeutic doses of GH in the absence of enhancers (Daugherty et al., 1988). The low bioavailabilities of GH and GHRH from the nasal mucosa would make treatment very expensive, but nasal delivery is still being pursued for administration to children.

Calcitonin is a calcium-regulating hormone secreted from the mammalian thyroid gland. It has a molecular weight of 3418 and contains 32 amino acid residues. It is a potent, natural inhibitor of bone resorption and has therapeutic uses for the treatment of osteoporosis and Paget's disease (Pontiroli and Pozza, 1989). Salmon calcitonin is commercially available as a nasal spray and in clinical trials has been effective in reducing back pain in patients with post menopausal osteoporosis and in increasing bone mineral content (Vega et al., 1989). The use of carbopol (0.1% w/v) has been observed to increase the hypocalcaemic effects of [Asu^{1,7}]-eel calcitonin in rats but the mechanism of enhancement is unclear (Morimoto et al., 1985).

1.2.5 Nasal absorption of vaccines

The administration of proteinaceous antigens to the respiratory tract is being actively pursued as a means of inducing mucosal and systemic response and has recently been reviewed by O'Hagan and Illum (1990). Vaccines which are being evaluated for nasal administration include, influenza, parainfluenza, measles, polio, rhinovirus and respiratory synctial virus (Chien et al., 1989). In many cases, intranasal vaccine delivery has been found to evoke an immune response and provide protection against viral challenge. Drug carriers and immunological adjuvants, including liposomes (Torchilin et al., 1988; El Guink et al., 1989; Bowen et al., 1990), nasal gels (Bremecker et al., 1988) and cholera toxin B (Tamura et al., 1989), are being investigated as a means of improving nasal administration of vaccines. It is expected that an acceptable vaccine for nasal immunization against influenza may be marketed in the near future (O'Hagan and Illum, 1990)

1.3 THE ANATOMY AND FUNCTION OF THE NOSE

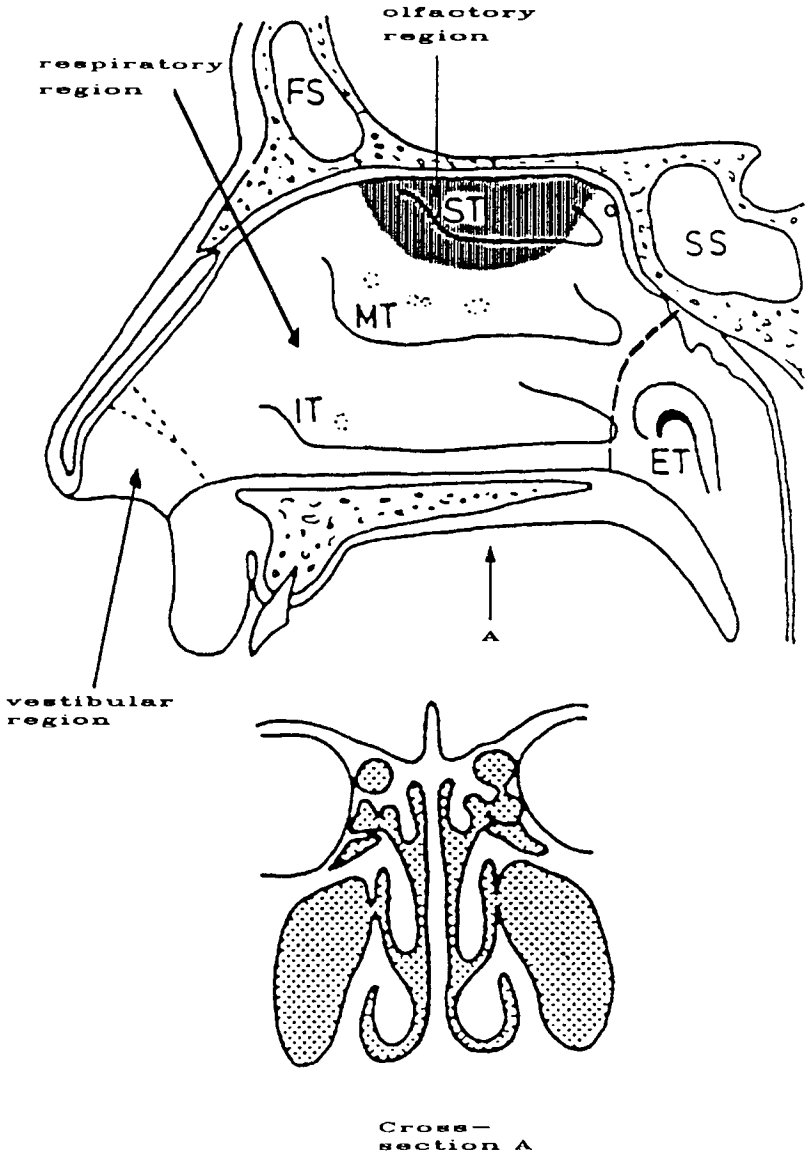
In order to study intranasal drug delivery, it is necessary to understand the anatomy and physiology of the nose and how these factors may influence drug absorption. A description of the salient features of the nose will be described in this section, based upon the work of Mygind (1979), Proctor and Anderson (1982), Geurkink (1983), Chien et al. (1989) and Tortora and Anagnostakos (1990).

The nose has a visible, external position consisting of a supporting framework of bone and cartilage covered with skin, which is lined with a mucous membrane. On the undersurface of the external nose are two openings to the face called the nostrils. In addition there is an internal non-visible part of the nose. This is a large cavity in the skull that lies inferior to the cranium and superior to the mouth. The walls and roof of the cavity are formed by the nasal conchae bones and the floor by the soft and hard palate. The nose is additionally subdivided into two right and left halves by a vertical partition called the nasal septum.

The nose is specialised to perform three distinct functions. Firstly, and perhaps most importantly in man, is the modification of inspired air, in order to protect the lower airways. This involves filtration of particulates and noxious agents from the air and their subsequent removal from the nose, and additionally, warming and humidification of the ambient air entering the nose. Close contact between the air flow and the nasal secretions at the mucosal surface occurs in the narrow airways. This provides the media for rapid water and heat-exchange, and serves as a trap for airborne-particulates, which may then be removed by mucociliary clearance. Its other roles include receiving olfactory stimuli and providing large, hollow, resonating chambers for speech sounds.

Air entering the nostrils will pass through three regions of the nose. These are termed the nasal vestibule, the respiratory region and the olfactory region. They each serve different physiological functions, and are positioned above and behind each other, as illustrated in figure 1.1. Entrance to the nose is via the funnel-shaped nasal vestibule which serves as a baffle system and provides the first filtration of airborne particles. Traversing the nasal vestibule there is a transition in the lining of the vestibular passages from skin, containing numerous hairs, to a region of squamous epithelium, which leads subsequently to a region of ciliated, columnar secretory lining.

Figure 1.1 A schematic diagram of the lateral wall of the nasal cavity and a cross-section at position A, through the middle of the nasal cavity. Based upon the work of Mygind (1979) and Proctor (1982). Where the dotted line represents the termination of the vestibular region at the nasal valve and the dashed line represents the position of the nasopharynx. IT, MT and ST are the inferior, middle and superior turbinates respectively. FS and SS are the frontal and sphenoidal sinus and ET, is the orifice of the Eustachian tube.



In this anterior part of the nose lies the nasal valve, which is an area of the lateral nasal wall and the adjacent septum, just before the beginning of the middle and inferior turbinates. The nasal valve is a slit-shaped structure, with a cross-sectional area of about 0.3-0.4 cm^2 at its narrowest point. It is the smallest passage for total respiratory airflow in the entire respiratory tract. The posterior termination of this feature is illustrated in figure 1.1 as a dotted line.

Beyond the nasal valve the passageways rapidly expand, to a cross-sectional area of some 1.3 cm^2 on each side. At this point the airways change direction by nearly 90 degrees. The result of these two phenomena, is that most aerosols and particles deposit in the anterior part of the nose (Proctor, 1985). Consequently, larger drug particles may lodge forward of the respiratory or olfactory region and therefore, may not be subject to absorption. Other insoluble particles may pass this point, but be removed from the nasal cavity by the mucociliary clearance mechanism. From this latter region a drug molecule introduced as a vapour or a particle, may successfully pass into the nasal secretions and subsequently be absorbed.

The region immediately beyond the nasal valve is the respiratory region, which is comprised of the convoluted, turbinate structures. The main nasal airways through this region are about 0.1-0.3 mm wide and are approximately 5-8 cm long. It is these narrow airways and folds of the turbinates, which provide the large surface area for drug absorption. In man, the total surface area of both nasal cavities is about 150 cm^2, providing a total volume of 15 mL (Mygind, 1979). The typical respiratory epithelium (ciliated columnar pseudostratified) consists of four dominant cell types. These are ciliated columnar cells, non-ciliated columnar cells, goblet cells and basal cells, which are illustrated in figure 1.2.

The third region of the nose is the olfactory region. This region lies above the middle turbinate, between the nasal septum and the lateral wall of the main nasal passage. It is represented in figure

Figure 1.2 A transmission electron microscopic diagram of the four cell types in nasal respiratory epithelium. I, represents non-ciliated columnar cells covered by microvilli. II, is a goblet cell containing mucous granules. III, is a basal cell and IV, represents ciliated columnar cells (Mygind, 1979)

1.1 as a shaded area and corresponds to an area of about 10 cm^2 in man. The olfactory passages are about 0.1-0.2 mm wide and are lined with olfactory epithelium. Histological evaluation indicates a pseudostratified columnar non-cilitated epithelium, comprising three cell types and is illustrated in figure 1.3. The cell types are basal cells, supporting cells and olfactory receptors cells, which are situated among the supporting cells. Drug delivery to the olfactory region of the nose, has been proposed as a possible means of delivering compounds directly into the brain *via* the olfactory neurons

Figure 1.3 A schematic diagram of the olfactory epithelium. (Chien et al., 1989)

and supporting cells (Gopinath et al., 1978; Madrid and Langer, 1991). Gopinath et al. (1978) showed that colloidal gold particles deposited on the olfactory epithelium of monkeys by nasal spray were absorbed by the olfactory neurons and migrated as far as the filia olfactoria. M. Hussain et al. (1990) have observed that the brain uptake of a cognition-enhancing drug, as indicated by a brain:plasma ratio, was comparable for both intranasal and intravenous administration. They suggested that a direct pathway to the brain was only significant for poorly absorbed solutes, such as peptides and metals.

The last part of the nasal airway is the nasopharynx, which extends from the posterior termination of the turbinates and arch of the nasal septum, backward and downward, to the border of the soft palate. At the nasopharynx, there is a transition from ciliated to squamous epithelium, although ciliated cells are present, particularly over the adenoid tissue. At this point the airways merge into one.

1.4 BIOLOGICAL BARRIERS TO NASAL DELIVERY OF PEPTIDE DRUGS

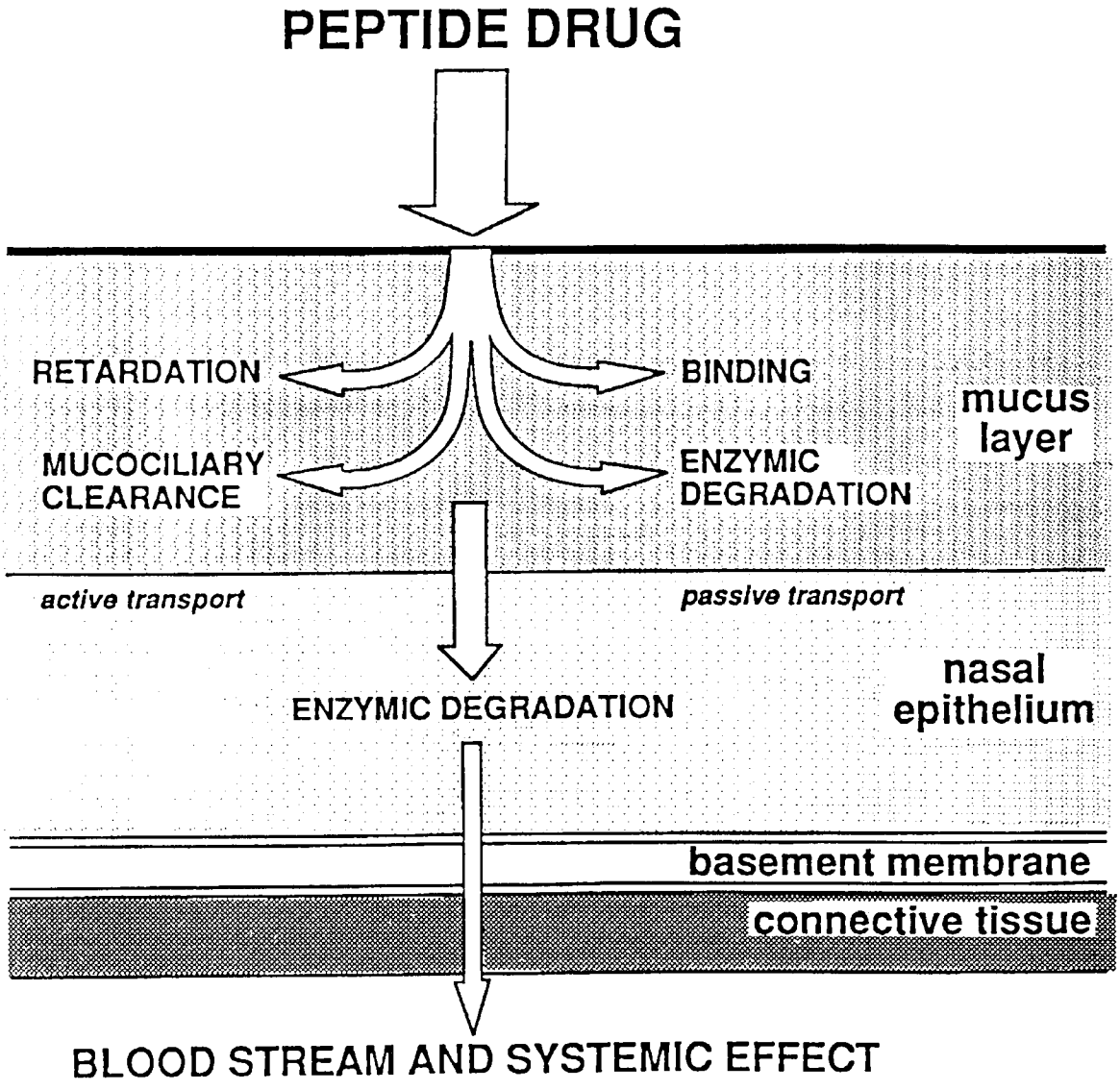
An intranasally administered peptide drug will encounter a number of biological barriers which may hinder systemic absorption. These can be considered as mucociliary clearance, nasal secretions, enzymes, hydrophobic membranes and transport processes (Wilson and Washington, 1990) and are illustrated in figure 1.4. The nature of these barriers will be discussed in the following sections.

1.4.1 Nasal Secretions

The first barrier that the drug must traverse is the layer of nasal secretions, which bathe the nasal mucosal surfaces. The composition of the secretions is a complex mixture of secretory material from mucous (goblet) cells, nasal glands, lachrymal glands and transudate from plasma (Marom et al., 1984).

Nasal secretions are the interface between the nasal mucosa and the ambient air and as such have a major role in maintaining the normal nasal functions in man, of protecting the lower airways and olfaction (Widdicombe and Wells, 1982). They also serve to prevent water loss

Figure 1.4 A schematic diagram of the biological barriers to peptide drug delivery.



from the nasal mucosa. The secretions protect the lower airuays in the first instance, by providing a media for exchange to occur across. This permits the transfer of heat and water to the inspired air (Litt, 1984) and simultaneously aids the removal of noxious agents, due to the adhesive nature of mucus (Taylor, 1974). Trapped noxious agents may then be either attacked by agents present in the secretions, such as immunoglobulins and bactericidal lysozyme, or be removed by mucociliary clearance. The fluid is essential both as a reservoir for protective agents and for efficient ciliary clearance from the nose (Sade et al., 1970). It has also been suggested that the secretions may aid olfaction, by either modifying the ambient air entering the olfactory region, or, by affecting the penetration of odours and their actions on the sensory cells (Widdicombe and Wells, 1982).

The properties of nasal secretions which enable it to perform its physiological function may also limit peptide drug absorption. This could be envisaged as retarding the diffusion of the peptide as a result of interactions with components of this layer, such as the glycoprotein molecules, or as a consequence of binding and clearance.

In a normal, healthy human the quantity of nasal secretion is scarce, the layer being typically 5 microns thick (Hilding et al., 1973). It is therefore difficult to obtain large quantities of the secretion that are representative of mucus *in vivo* fmr characterisation. Larger quantities of nasal secretions are produced in a variety of disease states including allergy, common cold and in those suffering from the genetic disorder cystic fibrosis, but they are not normal and are probably contaminated with inflammatory products and bacteria (Melon, 1969; Tremble, 1949; Hilding et al., 1973; Lorin et al., 1976; Marom et al., 1984).

When examined microscopically *in situ*, the nasal secretions are observed to possess a two-layer composition. There is a watery (sol) layer immediately adjacent to the mucosa, called the periciliary layer. The precise source of this layer is uncertain although it is believed to derive from the epithelium by a process of active

transport under the control of a chloride pump. The mucus (gel) layer lies in strands, or rafts, upon the periciliary layer (Widdicombe and Wells, 1982).

Information about the nature of nasal secretions has been obtained using a variety of techniques. These include nasal suction (Hilding, 1972), aspiration (Majima et al., 1988) and nasal wash techniques (Powell et al., 1977). An alternative approach has been to collect the secretions onto an absorbent such as filter paper (Lorin et al., 1972) and cellulose sponges (Wilson and Allansmith, 1976) introduced into the nose and then to analyse the fluid eluted from the absorbent. A few workers have used nasal provocation tests methacholine, histamine, atropine sulphate and allergens to evoke secretion (Brofeldt et al., 1986; Pelikan and Pelikan-Filipek, 1988; Raphael et al., 1988). Finally, there is an interest in using cultured nasal cells as a source of secretions for subsequent analysis (Boat et al., 1974; Patow et al., 1984a and b).

Some reviews have used data from nasal and tracheobronchial secretions interchangeably (Widdicombe and Wells, 1982). The description in this thesis is based on information for nasal secretions derived from one of the collection methods described above and also from histochemical analysis of nasal epithelium.

The major constituent of nasal secretions, comprising between 90 and 97%, is water. The other components are 2-3% glycoproteins, 1-2% salts, lipids, a variety of other proteinaceous material including immunoglobulins, enzymes and inhibitors, DNA and inflammatory mediators (Tremble, 1949; Mygind, 1979; Marom et al., 1984a and b; Jeffery, 1987).

The predominant inorganic components of human tracheobronchial secretions are sodium, chloride and calcium, in similar quantities to serum (Roussel at al., 1978). The ionic composition of human nasal secretions has been determined in secretions collected on to filter paper, inserted into the nasal cavity of healthy humans (Melon, 1969;

Lorin et al., 1972; Lorin et al., 1976). The concentrations of sodium, potassium, calcium and chloride ions determined by this method are presented in table 1.2. The mean values are similar to those reported for tracheobronchial secretions in laryngectomised patients (Richardson and Phipps, 1978).

Lipids in nasal secretions do not appear to have been studied. Their presence is expected as lipids have been determined in gastric mucus (Murty et al., 1984), salivary mucin (Slomiany et al., 1988) and tracheobronchial mucus (Woodward et al., 1982). Tracheobronchial secretions collected from human and hamster tracheal explants, have been shown to actively secrete neutral lipids, glycolipids and phospholipids, which were noncovalently bound to GPs (Clamp and Creeth, 1984; Chul Kim and Singh, 1990). Lipid interaction with GPs may influence the physical characteristics of mucus (Murty et al., 1984; Sarosiek et al., 1984).

Table 1.2 The ionic composition of human nasal secretions from the work of a, Melon (1969), b, Lorin et al. (1972) and c, Lorin et al. (1976). Where S.D. is the standard deviation and n, is the number of subjects tested.

ion	concentration (mM) mean	range	S.D.	n
sodium	128[a]	121-136	-	40
	150[b]	98-225	32	10
	140[c]	-	28	15
potassium	17[a]	12-25	-	40
	41[b]	23-68	18	10
	17[c]	-	8	15
calcium	5[a]	3-7	-	40
	8[b]	3-14	4	10
	6[c]	-	3	15
chloride	139[a]	129-150	-	40

Non-mucin proteins present in nasal secretions may arise from plasma transudation, or by active secretion (Raphael et al., 1988; 1989). Albumin is believed to be present as a result of plasma transudation and is the most prominent plasma protein (Mygind, 1979). Nasal secretions contain several classes of immunoglobulin, including IgA, IgG and in smaller amounts IgE (Remington et al., 1964; Lorin et al., 1972; Platts-Mills, 1979). IgA is the major immunoglobulin in nasal secretions, accounting for 20-50% of the total protein (Mygind, 1979). The concentrations of IgA are much higher than in serum, due to the synthesis and release of secretory IgA (sIgA) by local plasma cells in the lamina propria (Clamp and Creeth, 1984). Some IgG and IgE, is also believed to be synthesised locally in the nose (Lorin et al., 1972; Platts-Mills, 1979; Marom et al., 1984a and b)

Other actively secreted proteins found in nasal secretions are lactoferrin, lysozyme and kallikrein (Boat et al., 1974; Raphael et al., 1989). A number of other proteins with enzymatic properties have been detected in nasal secretions by Schorn and Hochstrasser (1979) including lactate dehydrogenase (LDH) and leucine aminopeptidase. The reason for these enzymes is unclear. Several protease inhibitors have been reported in nasal secretions by Hochstrasser (1983) and are proposed to moderate the action of lysosomal proteinases released by polymorphonuclear-leucocytes. These enzymes are known to cause a detrimental effect on nasal mucous membranes and cells.

1.4.1.1 Mucus glycoproteins.

It is the glycoprotein molecules (GPs) that are responsible for the gel forming, viscoelastic properties of nasal secretions. They are a distinct group of high molecular weight glycoconjugates, containing oligosaccharide side-chains attached to a protein core (Reid and Clamp, 1978).

Mucins are characterised by their high proportion of carbohydrate, which is typically greater than 70% and the type of sugars incorporated in the oligosaccharide chains (Allen, 1983). Sugars

typically present in mucin, are galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine and sialic acids. Only traces, if any, of mannose are found and no uronic acids. Mannose is the principal sugar in serum and membrane glycoconjugates and uronic acids are found in proteoglycans. In the protein core, the amino acids serine, threonine, proline and glycine predominate, whilst aromatic and sulphur-containing amino acids are deficient (Gallagher and Corfield, 1978). The linkage between the protein core and the carbohydrate chains, is via an O-glycosidic linkage, joining N-acetylgalactosamine to either serine or threonine. An analogy is often made between this structure and a 'bottle brush', where the carbohydrate chains represent the bristles and the protein core represents the central wire support.

Nasal GPs (NGPs) secreted from human nasal turbinates *in vitro* have been shown to have high molecular weights, covering a broad range of values from 0.4 x 10^6 to 20 x 10^6 Da, as determined by gel filtration of radiolabelled GPs (Patow et al., 1984a; 1984b). Patow et al. (1984b) determined that the NGPs had the characteristic GP composition, comprising 80% carbohydrate and 20% protein, and were of uniform acidic charge. The presence of negatively charged and neutral GPs on the nasal epithelium has been reported by Taylor (1974). The constituent carbohydrates, expressed as molar ratios relative to hexosamines were: hexosamines (1.0); galactose (0.87); N-acetylneuraminic acid (0.38); and fucose (0.3). The same rank order of carbohydrate constituents was reported in secreted NGPs in culture by Boat et al. (1974) and in nasal secretions induced by methacholine and histamine (Brofeldt et al., 1986).

The amino acid composition of NGPs has been determined by Boat et al. (1974) with an amino acid analyser, after acid hydrolysis of GPs secreted by cultured nasal polyps from patients with allergic rhinitis and cystic fibrosis. Serine was found to be the most abundant hydroxyamino acid rather than threonine, and relatively little proline was present. This is in contrast to other human respiratory GPs, where threonine predominates and proline is the third most

abundant amino acid (Woodward et al., 1982). An amino acid analysis of GPs from normal nasal tissue has not currently been reported. Hilding et al. (1973) found that proline, glutamic acid and glycine were the most abundant amino acids in normal nasal mucus. The study, however, used the whole nasal secretion, rather than purified GPs, which would have contained non-mucin protein.

The polymeric structure of NGPs is undetermined due to the scarcity of the secretion. The following section will review the information available from other mucins.

The overall polymeric structure of GPs is a subject of some controversy (Roussel et al., 1988). Two models have been proposed for the tertiary structure of mucins. The first, proposed by Allen (1983) for pig gastric mucus, is known as the 'windmill' model. The GP molecule is proposed to comprise four glycosylated enzyme-resistant subunits, joined centrally to a link-protein of molecular mass 70 kDa by disulphide bonds. This was derived from physical studies, demonstrating that the molecule was spherical, and from proteolysis and chemical reduction of the GP molecule. The second, and currently more favoured, model proposes that the mucin molecules are polydisperse, linear, flexible thread-like molecules, in which the glycopeptide subunits are linked end-to-end via disulphide bonds. This is proposed from electron microscopy of gastric, bronchial and cervical mucus (Sheehan et al., 1986; Hutton et al., 1988; Thornton et al., 1990). Robertson et al. (1989) dispute this model as it does not reconcile with the experimental observation that a putative 'link' peptide (or glycopeptide) seems to be present in many highly purified mucus glycoproteins.

The types of interaction involved in mucus gel formation are still uncertain. Disulphide bonds have been implicated in maintaining the gel structure of bronchial mucus (Roberts, 1976), whilst other workers maintain that respiratory mucus is an ensemble of physically entangled, randomly-coiled GPs, forming a loose network of variable density (Verdugo et al., 1983). Carlstedt et al. (1985) have

suggested that physical entanglement and noncovalent bonds between carbohydrate-rich regions of the mucins may be involved in gel formation.

1.4.1.2 Methods to determine the barrier properties of mucus

The apparatus used to investigate diffusion through mucus can be divided into two sections: those which use side-on three-compartment diffusion cells (Pfeiffer et al., 1981; Turner et al., 1985) and those which use capillary methods (Hughes, 1988; Cheema et al., 1988). In the three-compartment methodologies, the mucus is contained within two membranes, which are then inserted between the donor and receiver compartments. The diffusing molecule is added to the donor compartment and its appearance in the receiver compartment monitored. The disadvantages of this system include the relatively large quantities of mucus required to perform the experiment, the need to ensure that the apparatus is level (to eliminate pressure differences which would affect the results), and also the need to ensure the same stirring rate for each experiment performed.

The capillary method involves loading a glass capillary tube with mucus that has been mixed with a drug solution, and measuring the drug release from the mucus slab, across a semipermeable membrane, into a buffer solution. The concentration of the drug released into the buffer is determined at a set time and the diffusion coefficient determined. The prime advantage of this technique is the small quantity of mucus required, but care has to be taken to ensure that air bubbles are not introduced into the capillary.

A few recent reports have used fluorescent microscopy to determine mucus penetration (Henry et al., 1990; Radomsky et al., 1991). These allow the determination of very low levels of diffusant into the mucus layer. The major disadvantage is the uncertainty whether the observed fluorescence is due to the molecule of interest or to a fragment of the molecule bearing the fluorescent label.

1.4.2 Mucociliary clearance

In addition to penetration of the nasal secretions, inhaled particles in, or on, the mucus layer are continuously removed from the nose by the mucociliary clearance mechanism. This mechanism would clear peptide molecules from the nose to the gastrointestinal tract, where they would be degraded by resident proteases. Davis (1991) has proposed mucociliary clearance to be the major barrier (50%) to nasal peptide delivery.

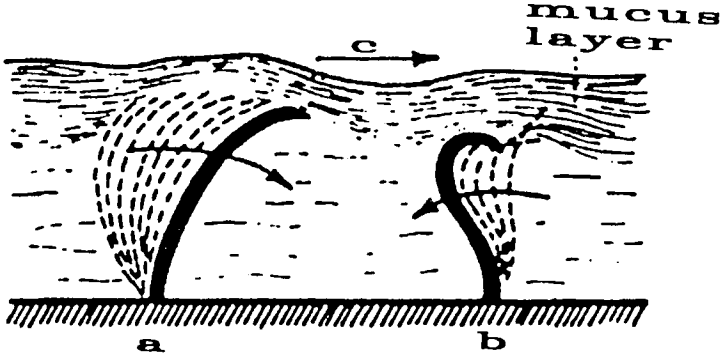
Successful clearance is dependent upon cilia, the periciliary layer and mucus. The cilia are long, thin, hair-like protrusions on the luminal surface of the epithelial cell. Respiratory tract cilia are about 5 - 6 μm in length and there are approximately 200 projecting from each epithelial cell, interspersed with short microvilli (Proctor, 1982b). 15-20% of the total number of cells in the nose are ciliated (Petruson et al., 1984).

The ultrastructure of respiratory cilia has been described by a number of workers to have a nine plus two pattern, typical of somatic cells. A cross section of a cilium shows an outer ring of nine pairs of microtubules surrounding two single microtubules. An outer doublet consists of an A and B subfibril with both an inner and outer dynein arm located on A subfibril with a radial spoke extending toward the central doublet, as shown in figure 1.5. Dynein is a complex protein, which has been demonstrated to have ATPase activity, necessary to breakdown ATP to provide energy for cilia movement (Satir, 1974). The microtubules are surrounded by a cell body which is an extension from the body of the respiratory epithelial cell.

Figure 1.5 A schematic diagram of a cross section through a respiratory cilium (Satir, 1974).

The beat of a single cilium consists of a forward (effective beat) and a slow return beat as illustrated in figure 1.6. The cilia beat synchronously in parallel ranks forming metachronical waves, which move the mucus layer in the direction of the effective stroke. The motion is dependent upon the microtubules sliding past one another, propelled by molecular bridges of dynein that project from one doublet to the next. The way in which coordinated beating is achieved is poorly understood but may be associated with neural innervation, chemical stimulation from hormones and the effects of ions such as calcium and potassium (Herzon, 1983).

Figure 1.6 A schematic diagram showing a, the effective and b, the recovery stroke of beating cilia. The direction of the mucus flow is indicated by c. The metachronical waves are illustrated in the lower figure.

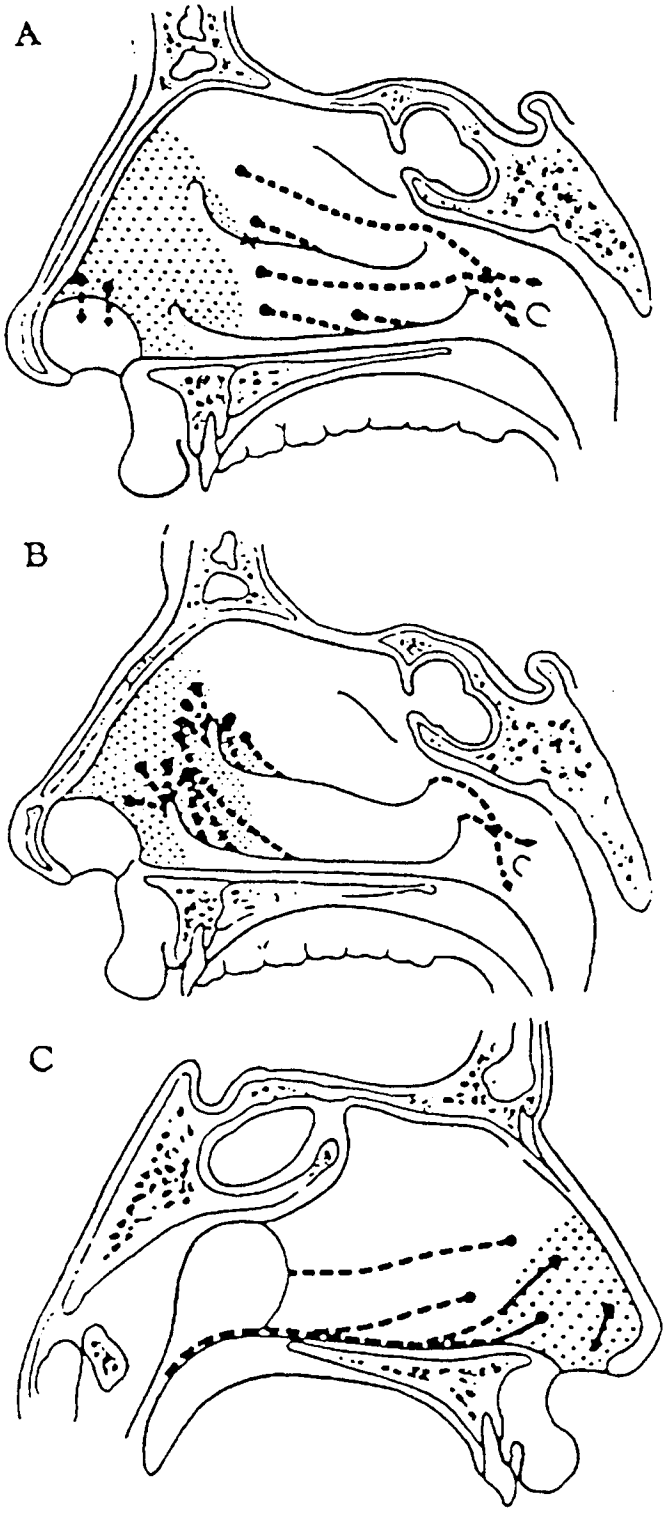


The cilia beat within the periciliary layer at a rate of 10 Hz in human nasal mucosa, as indicated by *in vitro* testing (Duchateau et al., 1985). Mucociliary transport is always in the same direction within a particular region of the nose. The patterns of mucociliary transport in the human nose are illustrated in figure 1.7. In the very front of the nose, secretion may become encrusted and removed digitally. From the lateral and septal walls, the mucus flow is downward and backwards, towards the pharynx, from where it is swallowed (Mygind, 1979). There is a large variation in the velocity of mucus transport in healthy human subjects from 1 to 20 mm min^{-1} with a average of 8.4 mm min^{-1} (Proctor, 1982b). The reason for such slow clearance is unknown.

To determine the nature and velocity of mucociliary clearance, and its individual components, a number of *in vivo* and *in vitro* methods have been pursued. In man, *in vivo* measurements of nasal clearance have been determined directly with the use of dyes, particles and saccharin tests and indirectly by external monitoring of intranasally administered radioactive or radio-opaque markers and has recently been reviewed by Schipper et al. (1991c). *In vitro* assessment has employed the frog palate to determine mucus transport rates (Giordano et al., 1977; Batts et al., 1989). The cilia beat frequency (CBF) has been determined *in vitro* using rat and chicken tracheal rings (Van de Donk et al., 1980a, 1980b; Duchateau et al., 1986; Batts, 1989), human cilia obtained from nasal brushings (Duchateau et al., 1985), human adenoids (Hermens et al., 1990) and cultured respiratory cells (Sanderson and Dirksen, 1986; Jorissen et al., 1989). An *ex vivo* method has been used by Levrier et al. (1989), whereby the solution was administered intranasally to guinea-pigs then the animals sacrificed, the tissue removed and the CBF determined *in vitro*.

There is some debate as to how useful CBF data and mucus transport times determined *in vitro* are as an indication of mucociliary clearance *in vivo*. A good example of this dilemma is the case of some preservatives used in nasal dosage forms. Levrier et al. (1989) reported that 0.1% mercurothiolate completely inhibited CBF *in vitro*,

Figure 1.7 A schematic diagram illustrating mucus flow in the human nose on a) the lateral wall in the ciliated area and the very front of the nasal cavity, b) on the lateral wall in the non-ciliated area and c) on the nasal septum.



but the same concentration was without effect *ex vivo*. Similarly, experiments performed with tracheal rings indicated that benzalkonium chloride (BC) and thiomersal (TH) would inhibit nasal clearance (Van de Donk et al., 1980b, Batts et al., 1990) whilst, experiments with the frog palate, containing a layer of mucus, suggested that BC and EDTA would inhibit mucociliary clearance and that TH would be well tolerated (Batts et al., 1989). Despite the *in vitro* contra-indications, *in vivo*, in man, it was found that 0.3 mL of 0.01% w/v thiomersal, 0.01% w/v BC and 0.1% w/v EDTA, each in 0.9% sodium chloride, were well tolerated by the nasal epithelium (Batts et al., 1991)

In addition to preservatives, a number of drug molecules have been observed to alter CBF *in vitro*, sometimes irreversibly. Those found to depress CBF include anaesthetics, antimicrobial agents, some antiallergics and propranolol, whilst others compounds have been observed to produce a excitory response (Duchateau et al., 1986; Hermens and Merkus, 1987). Encouragingly, insulin has been found to have no ciliostatic effect on adenoid tissue *in vitro* (Hermens et al., 1990) or on the mucus transport rate in the frog palate model (Gizurarson et al., 1990). A similar negative result was reported for the hexapeptide ORG2766 (Schipper et al., 1991a). Unfortunately, several of the enhancing agents, coadministered with peptide drugs have been observed to cause ciliostasis *in vitro*. Laureth-9 and deoxycholate have been found to be most ciliostatic, whilst glycocholate and taurocholate exerted only a mild effect (Hermens et al., 1990). The enhancing agent sodium dihydrotaurofusidate was also observed to cause ciliostasis *in vitro* at a concentration of 0.3% w/v. All of these compounds were also observed to arrest mucus transport on the frog palate, as did L-α-lysophosphatidylcholine (Gizurarson et al., 1990). The cyclodextrins, which are being actively investigated as absorption enhancers, exert only a mild effect on CBF (Schipper et al., 1991c).

The effect of altering mucociliary clearance on drug absorption will be discussed in section 5.1

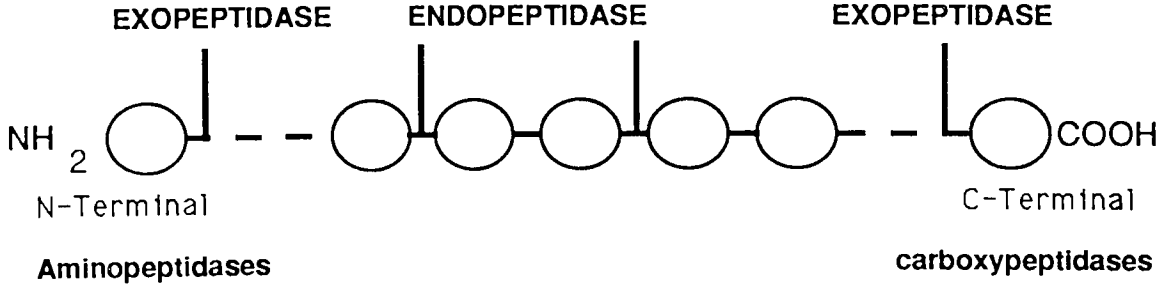
1.4.3 Enzymic activity against peptide drugs

Although it was originally proposed that the nasal mucosa was deficient in enzymic activity (Harris, 1986), it has been demonstrated that nasally delivered peptide drugs are susceptible to enzymic degradation by luminal, or membrane-bound proteases with an external active site (Hussain et al., 1990), in addition to intracellular degradation (Stratford and Lee, 1986).

The enzymes which are responsible for the degradation of peptides are termed either peptide hydrolases or proteases. They exert their effect by cleaving peptide bonds with the addition of water, and hence are classed as hydrolases. As such, they are placed in category 3.4 of the International Union of Biochemistry formal enzyme classification (Webb, 1984).

This broad group of enzymes is further subdivided into endopeptidases and exopeptidases according to their mechanism of action. Endopeptidases hydrolyse internal peptide bonds, whereas exopeptidases hydrolyse peptides either at, or very near, their N- or C- terminus. This is illustrated in figure 1.8. An exopeptidase whose action is directed towards the free amino end of a peptide, is termed an aminopeptidase, while one which attacks the free carboxy end, is referred to as a carboxypeptidase. The endopeptidases are further subdivided into five categories: serine; cysteine; aspartic; metallo and unknown action (Bond and Beynon, 1987).

Figure 1.8 Classification of proteases and their mechanism of action.

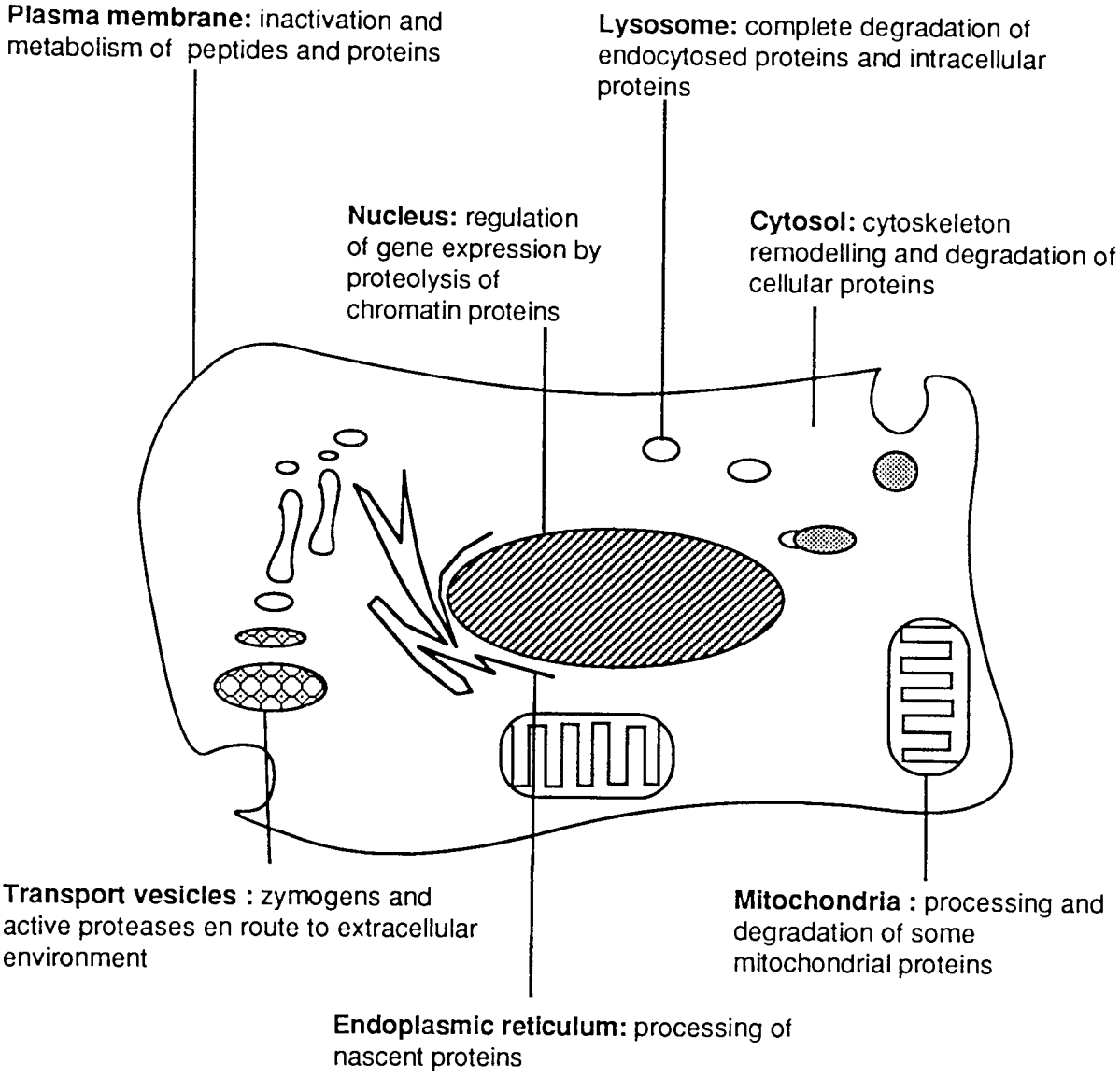


The most well known role of proteases is in the gastrointestinal tract, where proteins and peptides are degraded to amino acids, dipeptides and tripeptides, in order to provide the nutritional requirements of the body. Proteases are not merely digestive enzymes confined to this location and purpose, but additionally serve as mediators of physiological processes. As such, they have roles in activating zymogens to active enzymes, inactivation of biologically active molecules, intracellular proteolysis, and also in coagulation /fibrinolysis (Mahler and Cordes, 1971; Bond and Beynon, 1987).

These enzymes are distributed throughout the body to perform physiological functions, both luminally and intracellularly (Bond and Butler, 1987). The subcellular distribution and associated function of proteases is illustrated in figure 1.9. Consequently, peptide molecules are susceptible to degradation at different sites in the body. These include the site of administration, in the blood, liver and kidney and while crossing the vascular endophelia (Lee, 1986a) by a large number of different enzymes. From the large range of protease specificities, it is evident that a peptide molecule may be susceptible to attack by many, different enzymes, at any one of the peptide bonds along its chain. Hence, the protection of particular bonds, may not lead to significant increases in stability, or bioavailability.

The degradation of peptide drugs by proteolytic enzymes has been investigated only comparatively recently as most research has focused on digestion. The most widely used technique to study metabolism has been the use of homogenate tissue preparations. The weakness of this methodology is that the cellular organisation of the enzymes is destroyed and observed activity may consequently be nonphysiological. The techniques which have been applied to determine the proteolytic activity of the nose include nasal homogenates (Stratford and Lee, 1986; Zhou and Li Wan Po, 1991), cellular fractions (Choi and Lee, 1986; Yamamoto et al., 1988), perfusion techniques (Hussain et al., 1985b), nasal washes (Hussain et al., 1990) and whole animals (Faraj

Figure 1.9 Subcellular location and physiological function of proteases.



et al., 1990b). There are no reports, to date, of hydrolysis studies with cultured nasal or bronchial cells, although this technique may be used in the future (Audus et al., 1990).

1.4.4 The nasal epithelium

For a peptide molecule to leave the nasal cavity and produce a systemic effect, it must cross the nasal mucosa, comprised of epithelial cells (illustrated in figure 1.2), the lamina propria and the connective tissue (Mygind, 1979).

Epithelial membranes are selective, semi-permeable, hydrophobic membranes, which provide structure and permit the entry of required materials and the removal of waste products. There are essentially two ways in which drug molecules can traverse this layer and pass into the blood stream. The first is transcellularly, whereby the molecules are transported into and through epithelial cells, as a result of passive transport, active transport or membrane invagination. The alternative transport mechanism is intercellular, in which molecules pass directly into the blood stream via the junctions between the cells (Wilson and Washington, 1990).

Peptide drugs are generally high molecular weight, charged, and hydrophilic (unless terminal N- and C-termini are blocked by derivatisation). The latter two characteristics are unfavourable for transcellular transport and the former, makes diffusion through cell junctions difficult (Lee, 1988).

Active transport has been reported in the nasal cavity of rats for L-tyrosine and L-phenylalanine (Huang et al., 1985; Tengamnuay and Mitra, 1988b). Cremaschi et al. (1990) have proposed that the polypeptides elcatonin and adrenocorticotropic hormone are actively transported through the rabbit nasal mucosa by endocytosis. The existence of small peptide transporters in the nasal mucosa, as found and characterised in the intestine (Freidman and Amidon, 1990), has not been demonstrated in the nose to date.

The relationship between some physicochemical determinants, including size, charge, polarity and structure, and nasal drug absorption has been evaluated using whole animals (Gibson and Olanoff, 1987; McMartin et al., 1987), and isolated sheets of nasal mucosa mounted in Ussing chambers (Corbo et al., 1990), but is not well characterised.

Molecular weight is agreed by a number of workers to be an important parameter affecting nasal absorption (Fisher et al., 1987; McMartin et al., 1987; Donovan et al., 1990). Fisher et al. (1987) demonstrated a direct correlation between the log of the proportion of the nasally absorbed dose and the log of the molecular weight ($r = 0.996$), using a series of water soluble molecules ranging from 190 to 70,000 Da. Donovan et al. (1990) have also reported that absorption from the nasal cavity of rats exhibited a strong dependence upon the molecular weight of the polyethylene glycol, but could not identify a molecular weight cut off. McMartin et al. (1987), have proposed that nasal absorption is efficient up to a molecular weight of 1000

The mechanism of transport across the nasal mucosa is of some controversy. Hirai et al. (1981a) and Gibson and Olanoff (1987), have stated that lipophilicity is an important determinant of the transport rate. Gibson and Olanoff (1987) have proposed that the nasal mucosa is essentially a modified, lipophilic transport barrier, without evidence of aqueous pores, as indicated by the correlation between the nasal absorption of alkanoic acids and log partition coefficients, and the zero uptake of the uncharged, hydrophilic molecule mannitol. The rate and extent of nasal absorption of progesterone and its hydroxy derivatives has been observed to decrease as penetrant hydrophilicity increased *in vivo* and *in vitro* (Corbo et al., 1989a; 1989b; 1990). A similar effect was observed using a series of barbiturates administered to rats (Huang et al., 1985).

McMartin et al. (1987) have proposed that two mechanisms of drug transport are involved in nasal absorption. A fast rate, which was dependent upon lipophilicity and a slower rate, dependent on molecular weight, which nevertheless permits a high degree of absorption for low

molecular weight polar compounds. While Gibson and Olanoff (1987) have reported the absence of aqueous pores in the nasal mucosa other workers advocate their presence, and role, in the absorption of water soluble molecules (Hayashi et al., 1985; Fisher et al., 1987)

1.5 AIMS AND OBJECTIVES OF PRESENT STUDY

While the nasal route of administration has been shown to be successful for the delivery of some bioactive peptides, to optimise delivery it is important to define the nature and relative contribution of the factors which restrict systemic absorption from the nose. The aim of this project was to assess the importance of two of the biological barriers in the nose, namely the mucus layer and the enzymic barrier. Greater mechanistic information about the role of these two biological phenomena is fundamental to the successful design and development of nasal drug delivery systems for a plethora of bioactive peptides. The development of such delivery systems would make the widespread usage of peptide molecules in therapy and diagnosis a reality.

The first objective was to develop a representative model of *in vivo* mucus, in order to investigate the barrier that this gel presents to peptide delivery. The nature of the barrier was to be explored in terms of drug diffusion and drug binding. Any correlation between the diffusion and binding properties and the physicochemical properties of the diffusants was also to be investigated. Additionally, ways of modifying the mucus barrier were to be explored, employing absorption enhancers previously used in intranasal formulations.

Similarly, the initial aim of the enzyme studies was to establish suitable enzymic preparation methodologies which would be representative of the enzymic activity of the nasal tissue and luminal enzymes. Having established the model, the aim was to determine the degradation routes and relative stabilities of a series of chemically similar peptides, and to simultaneously probe the presence, and relative activities of different enzymes in the nasal mucosa and the lumen.

CHAPTER 2
ASSAY PROCEDURES

CHAPTER 2

ASSAY PROCEDURES

2.1 INSTRUMENTATION

2.1.1 Ultra-violet absorption

Ultraviolet (UV) absorption measurements were determined using a Cecil C292 Digital UV-spectrophotometer. UV scans were recorded with a Pye Unicam SP8000 Ultraviolet scanning spectrophotometer. Quartz cells, with 10 mm pathlength, were employed in both cases.

2.1.2 High-performance liquid chromatography (HPLC)

The HPLC system employed comprised an Altex model 110A pump, a Rheodyne 7120 or 7125 injection port fitted with a 100 µL loop and a Pye Unicam LC3 variable wavelength ultraviolet detector. Chromatography was performed on a 4.6 mm by 100 mm (internal length by column length) column packed with 5 µm ODS-Hypersil reversed-phase material. A guard column (4.6 x 10 mm) packed with the same material was used to increase the lifetime of the column. Chromatograms were recorded on either a JJ Instruments recorder or an Omniscribe D500 chart recorder. The loan of a Hewlett Packard 3390A recording integrator by Fisons Pharmaceuticals permitted the peak areas to be determined. This will be referred to as system A

The analysis of the majority of the metabolism experiments was performed on a Waters Chromatography station. This comprised a Waters 600E system controller, a WISP 712 autoinjector and a Waters 484 ultraviolet variable wavelength detector. Chromatography was performed on 4 mm by 250 mm Lichrospher 100 RP-18 end-capped column with 5 µm particles protected by a guard column packed with the same material. Chromatograms were collected and integrated on a Waters Chromatography 815 workstation. This will be referred to as system B.

2.1.3 Subsidiary

All pH measurements were determined with a WPA CD 660 Digital pH meter (3 decimal place display) connected to a Gallenkamp combination glass electrode. This was calibrated prior to use with Colourkey buffer solutions (BDH).

Three Sartorius balances were employed for weighing purposes. These were a 1601 MP8, an analytical A200S (4 decimal place) or a research R200D (5 decimal place)

A Kerry sonicator was utilized to aid dissolution, where required, and to degas HPLC mobile phases to be used with system A.

2.2 HPLC METHOD DEVELOPMENT

HPLC is a versatile analytical technique which is widely used in pharmaceutical analysis for the rapid, specific and sensitive detection of a range of molecules. It is particularly valuable in the analytical separation and quantification of closely related compounds, such as enantiomers, degradation products and metabolites. The pharmaceutical applications of HPLC have been reviewed by Li Wan Po and Irwin (1980), Irwin and Scott (1982) and Munson (1984).

The theoretical principles and practicalities of HPLC may be found in several texts and will only briefly be mentioned in this thesis. The components of an HPLC system are: the solvent delivery system; the injection system; the analytical column; the mobile phase and the detection and recording devices. In brief, a sample is applied to a solid-phase analytical column by means of an injection port and a pumped mobile phase. The sample then interacts with the column and is retained upon it. A combination of partitioning and adsorption between the stationary and mobile phases determines the separation and elution of the analytes. The sample is then eluted from the column and detected by the detection device.

Suitable assay procedures may be developed by modification of one, or more, of the components of the system. The initial step is the selection of an appropriate analytical column. The optimum detection and resolution, is then achieved by manipulation of the mobile phase composition in conjunction with selection of the most appropriate detection system. The separation may be quantified by means of mathematical parameters, which will be described later.

2.2.1 The analytical column

The analytical column has been described as the heart of HPLC and needs to be selected with care, as this will determine the nature of the mobile phase required. The majority of HPLC separations are performed on columns packed with porous silica-based material, with controlled particle sizes in the range 3-10 μm. The silica may be untreated or chemically modified.

Untreated silica possesses polar silanol groups (Si-OH) on its surface, which impart a polar nature to the adsorbent. This is exploited in normal-phase HPLC, where the separation is dependent on adsorption of solute from a relatively non-polar mobile phase by interaction with the polar silanol groups.

An alternative approach is to chemically modify the surface silanol groups to produce a non-polar absorbent, by chemical reaction with a long-chain hydrocarbon derivative. An example of this, is the reaction between the silanol groups and chlorosilane, which is used to form octadecylsilane (ODS, C_{18}) packing. ODS-bonded phase is probably the most widely employed stationary phase of this type and was selected for use in this study. The hydrophobic alkyl bonded phase permits the use of highly polar mobile phases in a process known as reversed-phase chromatography. In this mode, it is possible to separate polar compounds which would not be eluted from normal phase systems, with more polar compounds eluting from the column first, followed by non-polar compounds.

A major problem with bonded silica phases is that after chemical derivatisation, residual surface silanol groups may still be present. Their presence imparts a bifunctional nature to the surface of the absorbent with both acidic, polar regions (Si-OH) and non-polar regions present. Consequently, compounds which exhibit both hydrophobic and hydrophilic molecular regions, may interact with both types of functional groups, resulting in broad asymmetrical tailing peaks. Effects to suppress peak tailing, have focused on reducing these silanol interactions by modifications to the stationary and

mobile phases, by "end-capping" and the inclusion of organic modifiers respectively. End-capping is a secondary bonding reaction, between the bonded material (with residual Si-OH groups) and typically a short chain silane, which further removes the polar Si-OH groups. The use of organic modifiers will be discussed in section 2.2.2.

Silica-based bonded phase materials were operated at the recommended pH values between 2.5 and 7.0. Below pH 2.5, the column will be susceptible to loss of bonded phase, whereas above pH 7.0, the silica particles are liable to dissolve. The column performance may additionally be impaired by prolonged use and deposition of lipid and proteinaceous material on the stationary phase. In the case of the ODS columns this is indicated by reduction in solute retention times and deterioration in peak shape. This problem may be reduced by the incorporation of a guard column, packed with ODS, which can easily be repacked with fresh material when marked deterioration has occurred and by preventative washing of the HPLC system with distilled water and various organic:water mixtures, of increasing hydrohobicity, immediately after use.

2.2.2 The mobile phase

Modification of the polarity and pH of the mobile phase imparts selectivity to the system. The development of isocratic elution methods for different compounds was thus achieved by systematic, quantitative changes in solvent composition. Mobile phases of varying proportions of acetonitrile and water were employed to optimize peak resolution whilst maintaining a relatively short analysis times

The peak tailing introduced by residual Si-OH groups, may be partly resolved by the incorporation of an organic modifier, which undergoes strong polar interactions with Si-OH groups and therefore minimizes similar interactions with the solute of interest. Several of the compounds studied exhibited broad, tailing peaks. The incorporation of 0.1% v/v diethylamine at acidic pH to the mobile phase, appeared to improve the chromatography and was therefore included in most

analytical methods. Adjusting the pH affects the ionized form of the solute and may be manipulated to improve chromatography.

Mobile phases employed in reversed-phase HPLC contain a large aqueous component, which will contain dissolved gases. These gases may exert a deleterious effect on chromatography by out-gassing in the detector flow cell or the formation of UV-absorbing complexes with solvents (Bakalyer et al., 1978). Mobile phases were degassed by vacuum filtration, sonication or by a helium sparge prior to HPLC analysis.

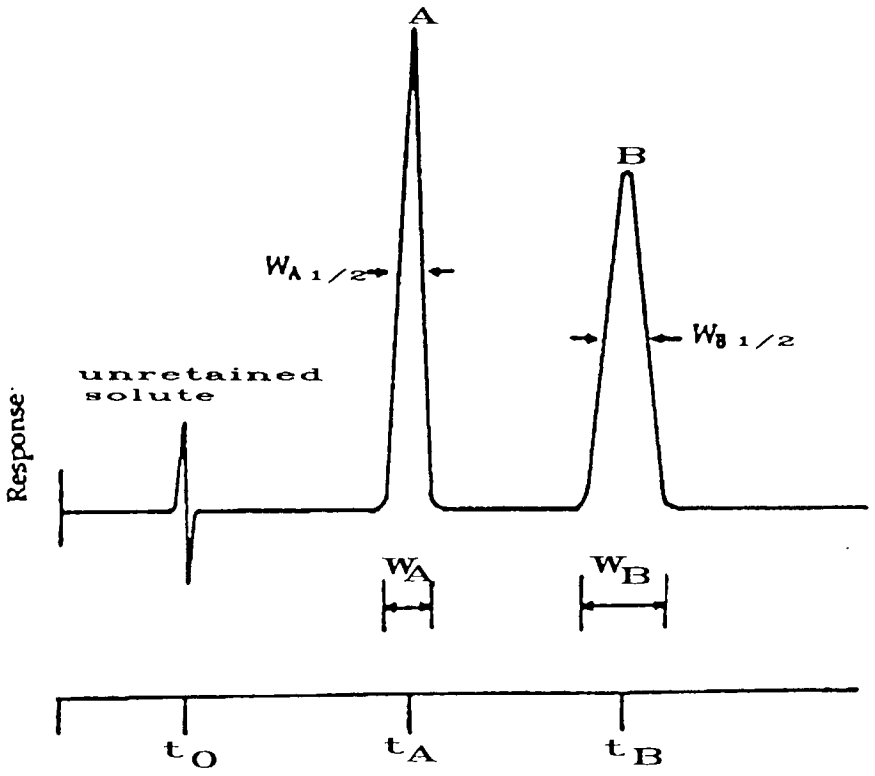
2.2.3 The detection system

Several detectors are available for HPLC use including UV-Visible, fluorimetric, refractive index and electrochemical. In this study UV detection was used exclusively. The use of this detector to determine the presence and concentration of analytes, requires a knowledge of the UV absorption characteristics of the compounds of interest. The UV spectra of each compound, and possible internal standards, were determined in the range 200-350 nm and the wavelength at which maximum absorbance occurred (λ_{max}) recorded. This wavelength was then selected for development of the analytical method.

2.2.4 Mathematical parameters

A HPLC system may be quantified by a number of mathematical parameters which define its chromatographic performance in terms of retention and resolution. A typical HPLC chromatogram for a two-component mixture is illustrated in figure 2.1 in which: t_O, t_A and t_B are the retention times of an unretained solute, component A and component B respectively. The parameter, t_O, is commonly recognised as the first disturbance in the baseline, usually observed as the solvent front, with which the unretained solute is considered to co-elute. The corresponding retention volumes (V_O, V_A and V_B) may be calculated from the mobile phase flow rate (F mL min^{-1}) in equation 2.1

Figure 2.1 Example chromatogram of a two-component mixture (from Li Wan Po and Irwin, 1980)



$$V_O = t_O F \qquad (2.1)$$

Where, V_O is the column void volume (or dead volume) and is a measure of the inter- and intra-particular pores.

The column capacity, k', is a measure of the sample retention and may be determined from equation 2.2:

$$k' = \frac{(t_A - t_O)}{t_O} \qquad (2.2)$$

For good isocratic separation, k' should be in the range of 1 to 10. Values outside this range should be avoided since values less than 1, are indicative of inadequate separation from the solvent front and

values greater than 10, are associated with long retention times and broadened peaks.

The number of theoretical plates, N, gives a measure of the column efficiency, which is dependent on the degree of band broadening relative to the time taken to elute. For the example chromatogram in figure 2.1, this may be defined by the equation:

$$N = 16 \, (t_A / W_A)^2 \qquad (2.3)$$

where W_A is the base width of peak A. N is usually expressed per metre of column, which is derived by dividing equation 2.3 by the column length in metres and should be in the range of 2500 to 10,000. Columns with large N values will provide narrow, better resolved peaks, than those with lower values. An alternative parameter to reflect column efficiency is H, the height equivalent of a theoretical plate where:

$$H = \frac{L}{N} \qquad (2.4)$$

and L is the column length in μm. Values for H should be within the range 25-100 μm.

The resolution of a system, R_s, is a measure of the efficiency of the separation and may be represented by equation 2.5:

$$R_s = \frac{2 \, (t_B - t_A)}{(W_A + W_B)} \qquad (2.5)$$

An R_s value of 1.0 indicates a satisfactory separation with approximately 2% overlap, whilst a value of 1.5 represents almost total separation.

2.3 HPLC METHODS DEVELOPED

The following sections describe the analytical HPLC methods which were developed to analyse compounds in subsequent work with mucus and peptide hydrolysis. The format used to illustrate each method includes: the chemical structure of the compound; a table of the HPLC conditions employed; a typical chromatogram; calibration curve with associated statistics; and derived HPLC parameters. The concentration of the internal standard (I.S.) given in the table of conditions, is the final concentration of injected I.S. in the sample. The final injected concentrations of the analytes are those given in the calibration graphs. The injection volume in each case was 100 µL.

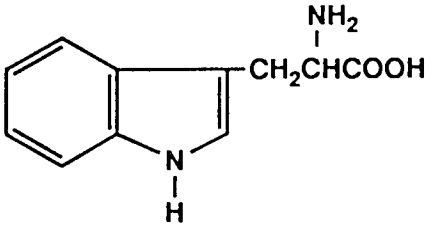
2.3.1 HPLC methods for use with mucus experiments

2.3.1.1 Tryptophan and phenylalanine

A number of analytical methods are available for the analysis of amino acids. Methods have utilised ion-exchange chromatography coupled with either post- or pre-column derivatisation with agents such as ninhydrin, o-phthalaldehyde (OPA) and fluorescamine, followed by absorbance or fluorescence detection (Hill et al., 1979; De Jong and Hughes, 1982; Hughes and Winterhaler, 1982; Bridlingmeyer et al., 1984). The derivatisation step increased sensitivity, but pre-column derivatisation can be tedious and the derivatised amino acids less stable. In post-column derivatisation peak resolution is reduced, as a consequence of the dead volume introduced by the derivatisation reaction chambers. The amino acids tryptophan (TRP) and phenylalanine (PHE) possess aromatic rings as shown in figure 2.2, which confer UV absorption. It was therefore decided to use reversed-phase HPLC and UV detection. The HPLC conditions employed are tabulated in table 2.1.

Figure 2.2 The chemical structure of a) tryptophan and b) phenylalanine

a)



b)

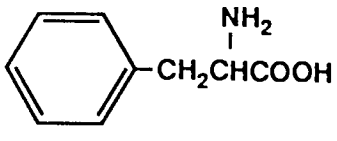


Table 2.1 HPLC conditions for the separation and detection of a) tryptophan (TRP) and b) phenylalanine (PHE)

a)

system	A
mobile phase	5% acetonitrile 0.1% diethylamine pH 3.5 with orthophosphoric acid
flow rate	1.5 mL min^{-1}
wavelength	265 nm
injection solvent	phosphate buffer (pH 7.4)
internal standard	PHE (5×10^{-4} M)
AUFS	0.08

b)

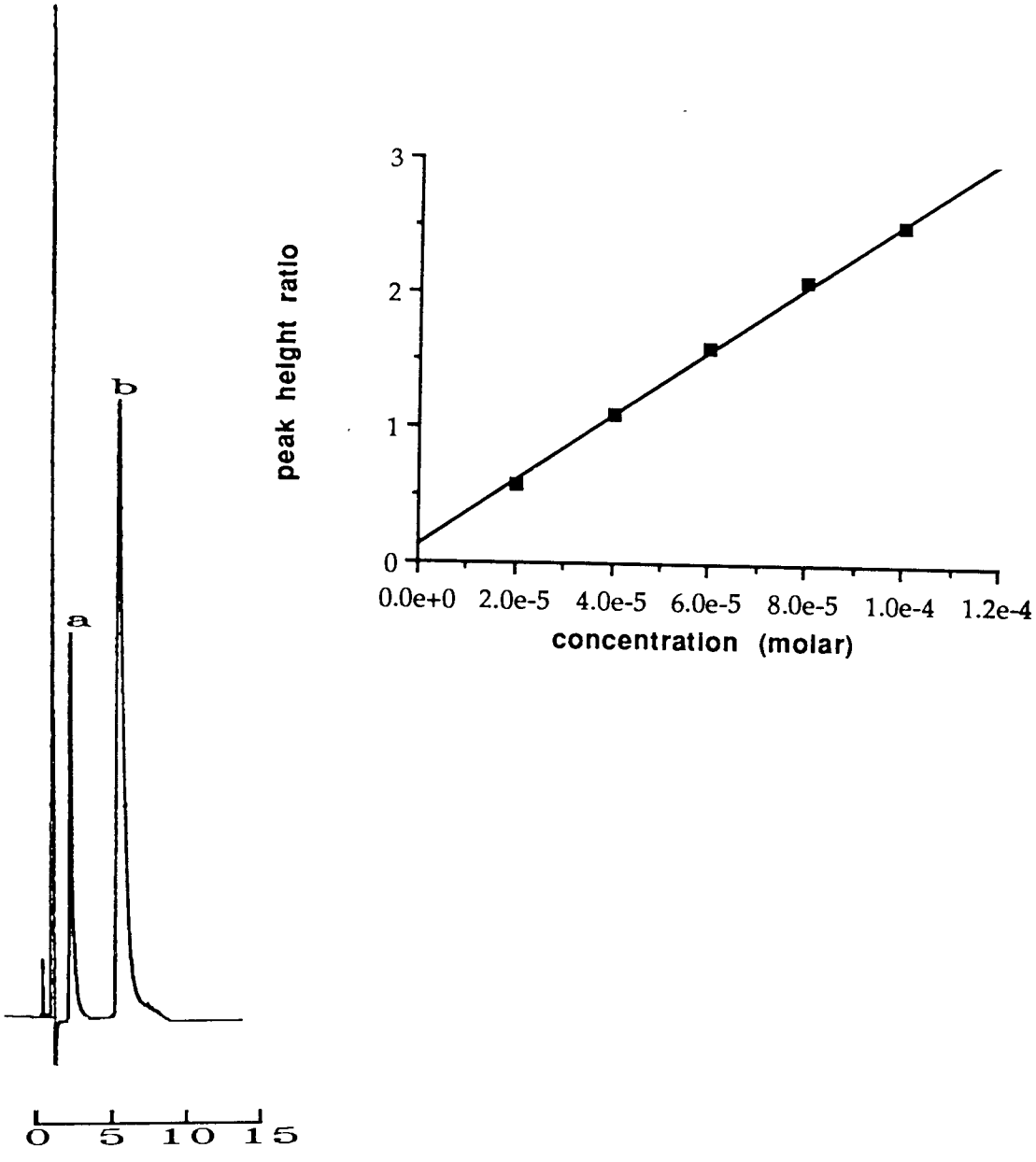
system	A
mobile phase	5% acetonitrile 0.1% diethylamine pH 3.5 with orthophosphoric acid
flow rate	1.5 mL min^{-1}
wavelength	265 nm
injection solvent	phosphate buffer (pH 7.4)
internal standard	TRP (5×10^{-4} M)
AUFS	0.08

Table 2.2 Calibration statistics for tryptophan (TRP) with 5×10^{-4} M phenylalanine (PHE) as I.S., and phenylalanine with 5×10^{-4} M tryptophan as I.S.

compound	slope ($\times 10^{-4}$)	intercept ($\times 10^{2}$)	correlation coefficient
TRP	2.4497	10.979	0.999
PHE	0.1974	6.9021	0.999

Figure 2.3 Examples of a) a typical chromatogram of tryptophan (peak b, 5 x 10^{-4}M) , with phenylalanine (peak a, 5 x 10^{-4}M) as internal standard and b) typical calibration curve for tryptophan with 5 x 10^{-4} M phenylalanine as I.S. and c) typical calibration curve for phenylalanine with 5 x 10^{-4}M tryptophan as I.S., using system A. For calibration curves points are the mean ± sem of at least two determinations.

a) b)



c)

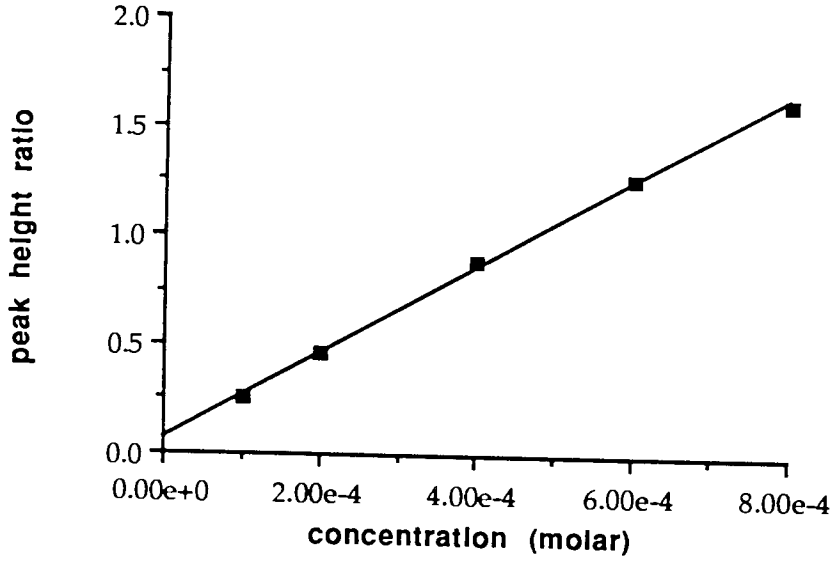


Table 2.3 HPLC parameters for tryptophan (TRP) and phenylalanine (PHE). R_s is the resolution of TRP from PHE.

compound	t_R (min)	k'	N (m^{-1})	H (μm)	R_s
PHE	2.0	1.0	2560	390	4.67
TRP	5.5	4.5	4840	206	

2.3.1.2 Tryptophylglycine

Figure 2.4 The chemical structure of tryptophylglycine.

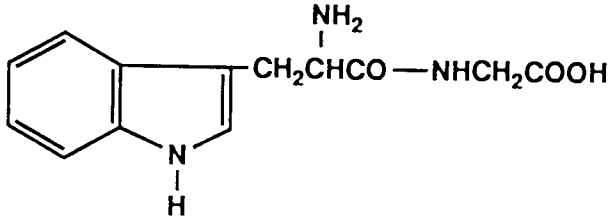


Table 2.4 HPLC conditions for tryptophylglycine (WG).

system	A
mobile phase	4% acetonitrile 0.1% diethylamine pH 3.5 with orthophosphoric acid
flow rate	1.5 mL min^{-1}
wavelength	270 nm
injection solvent	phosphate buffer (pH 7.4)
internal standard	TRP (5×10^{-4} M)
AUFS	0.08

Table 2.5 Calibration statistics for tryptophylglycine (WG)

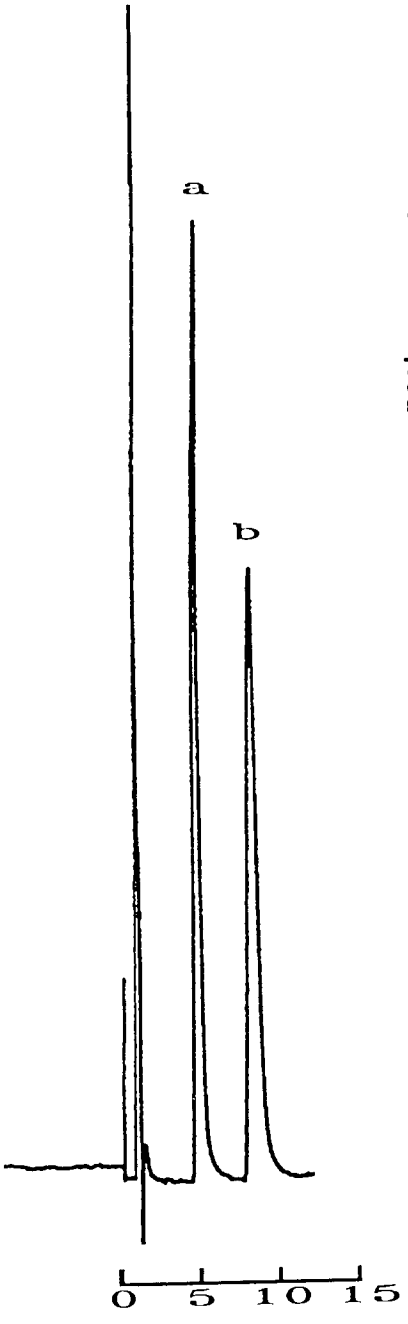
compound	slope ($\times 10^{-4}$)	intercept ($\times 10^{2}$)	correlation coefficient
WG	1.9696	-6.870	0.999

Table 2.6 HPLC parameters for tryptophylglycine (WG) with tryptophan (TRP) as internal standard. R_s is the resolution of WG from TRP.

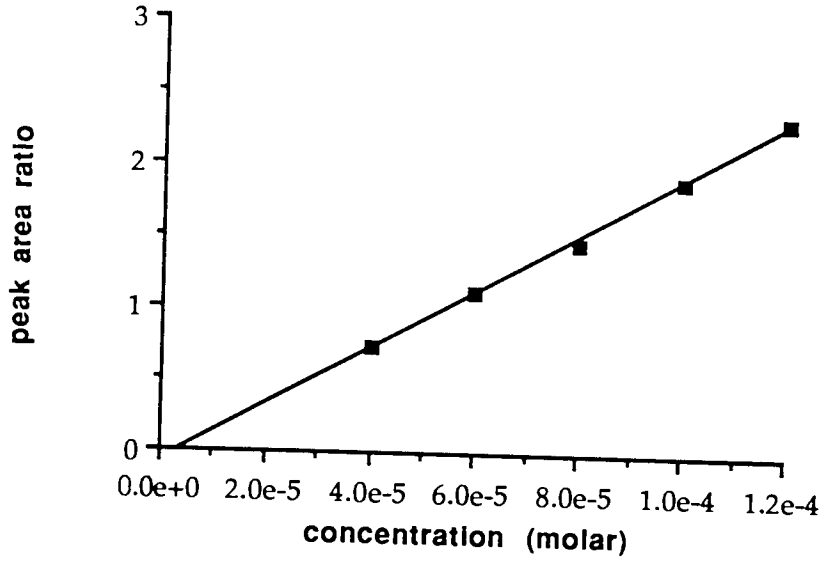
compound	t_R (min)	k'	N (m^{-1})	H (μm)	R_s
TRP	4.5	3.5	12960	77.2	4.67
WG	8.0	7.0	10240	97.6	

Figure 2.5 Examples of a) a typical chromatogram tryptophylglycine (peak b, 6 x 10-5 M) with tryptophan (peak a, 5 x 10-5 M) as internal standard and b) typical calibration curve, using system A. For calibration curves points are the mean ± sem of at least two determinations.

a)



b)



2.3.1.3 Tryptophylglcylglycine

The HPLC conditions were as described in table 2.4

Figure 2.6 The chemical structure of tryptophylglycylglycine.

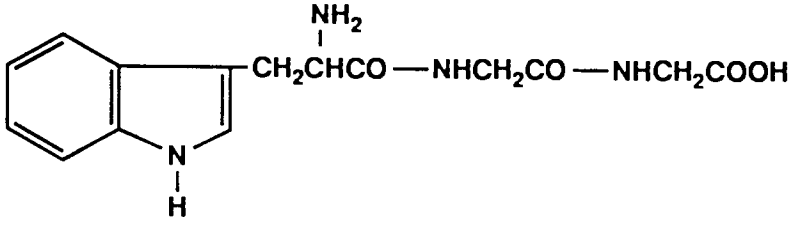


Figure 2.7 Examples of a) a typical chromatogram of tryptophylglycylglycine (peak b, 6 x 10-5 M) with tryptophan (peak a, 5 x 10-5 M) as internal standard and b) typical calibration curve, using system A. For calibration curves points are the mean ± sem of at least two determinations.

a) b)

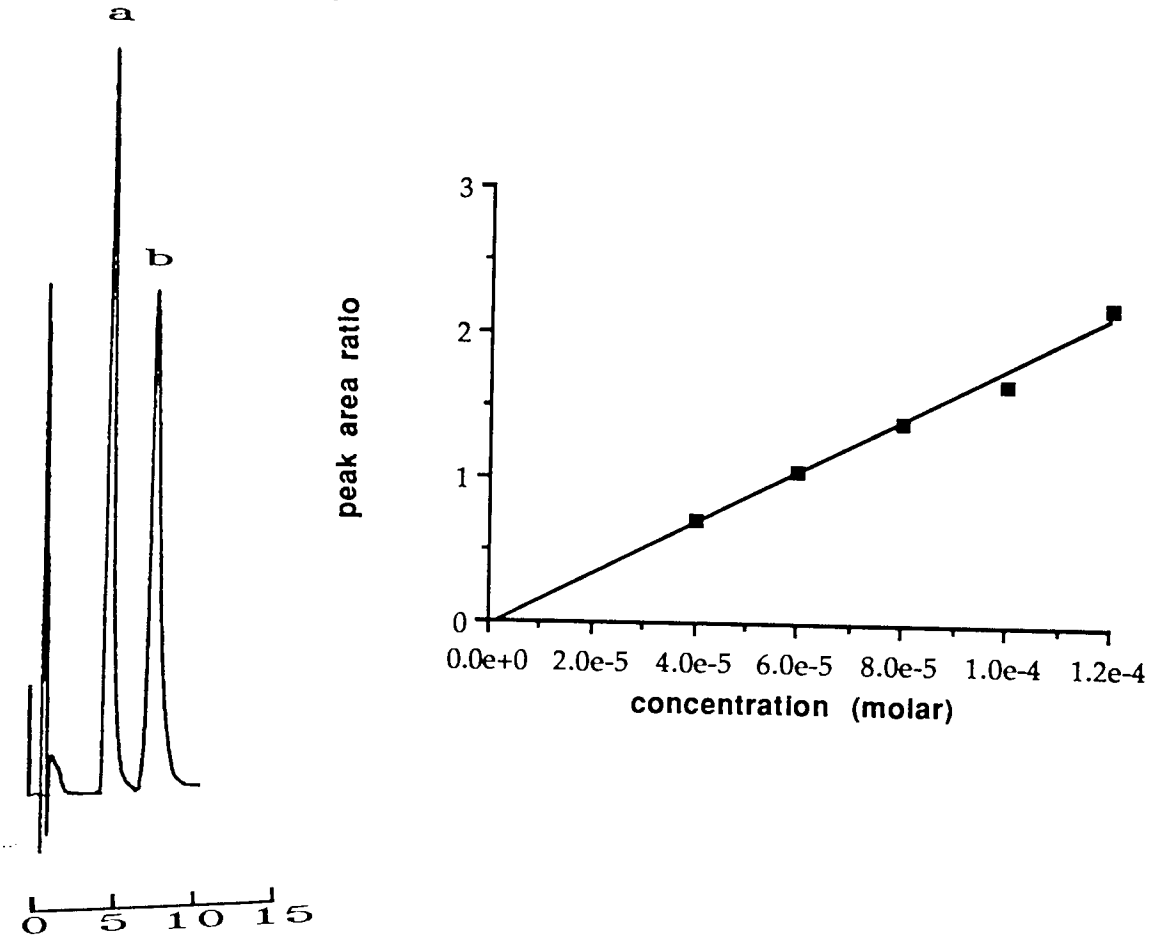


Table 2.7 Calibration statistics for tryptophylglycylglycine (WGG)

compound	slope (x10-4)	intercept (x10²)	correlation coefficient
WGG	1.811	-3.312	0.993

Table 2.8 HPLC parameters for tryptophylglycylglycine (WGG) and tryptophan (TRP) as internal standard. R_s is the resolution of WGG from TRP.

compound	t_R (min)	k'	N (m^{-1})	H (μm)	R_s
TRP	5.0	5.0	4000	250	2.00
WGG	7.5	6.5	4000	250	

2.3.1.4 Benzoic acid

The assay was developed from the method of Gupta (1977)

Figure 2.8 The chemical structure of benzoic acid

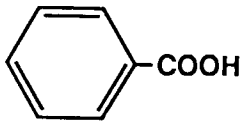
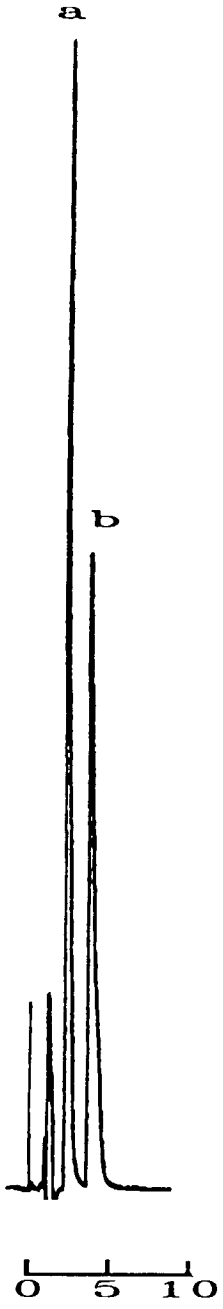


Table 2.9 HPLC conditions for benzoic acid (BA)

system	A
mobile phase	acetonitrile : 0.02M KH_2PO_4, 5:95 pH 6.2 with 0.1M sodium hydroxide
flow rate	1.0 mL min^{-1}
wavelength	240 nm
injection solvent	phosphate buffer (pH 7.4)
internal standard	salicylic acid (1 x 10^{-4} M)
AUFS	0.16

Figure 2.9 Examples of a) a typical chromatogram of benzoic acid (peak a, 1.2 x 10-4 M), with salicylic acid (peak b, 1 x 10-4 M) as internal standard and b) typical calibration curve, using system A.

a)



b)

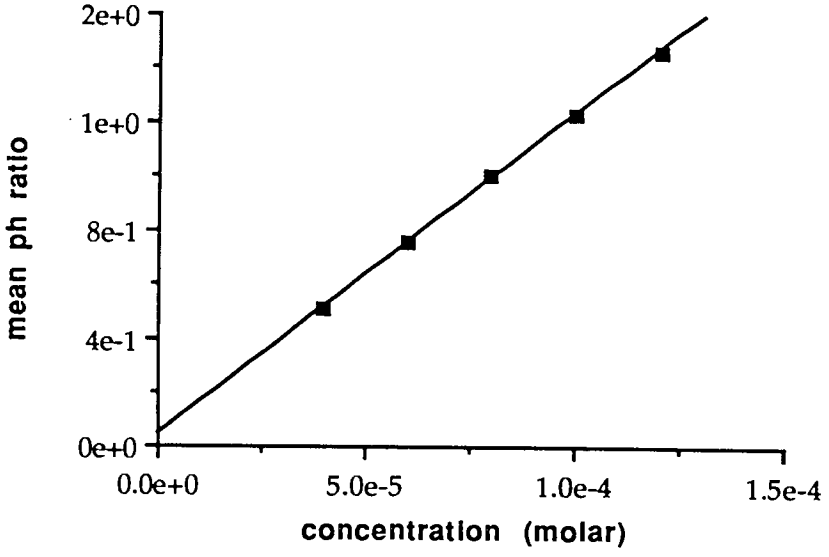


Table 2.10 Calibration statistics for benzoic acid (BA)

compound	slope (x10-4)	intercept (x10²)	correlation coefficient
BA	0.1192	4.14	1.000

Table 2.11 HPLC parameters for benzoic acid (BA) with salicylic acid (SA) as internal standard. R_s is the resolution of BA from SA.

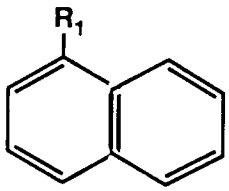
compound	t_R (min)	k'	N (m^{-1})	H (μm)	R_s
BA	2.5	1.5	1778	562	2.29
SA	4.5	3.5	3240	308	

2.3.1.5 Propranolol

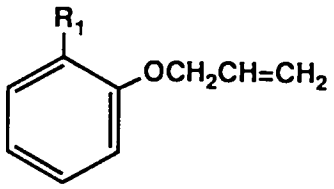
The HPLC methods for propranolol were developed from the work of Belaid (1986).

Figure 2.10 The chemical structure of the three β-blockers propranolol, oxprenolol and atenolol.

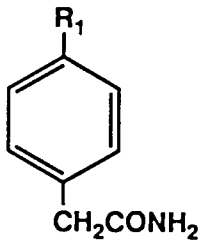
PR



OX



AT



R_1 = $-OCH_2CH(OH)CH_2NHCH(CH_3)_2$

Table 2.12 HPLC conditions for propranolol (PR)

system	A
mobile phase	40% acetonitrile 0.1% diethylamine pH 2.5 with orthophosphoric acid
flow rate	1.0 mL min^{-1}
wavelength	290 nm
injection solvent	phosphate buffer (pH 7.4)
internal standard	methylparaben (1 x 10^{-4} M)
AUFS	0.08

Figure 2.11 Examples of a) a typical chromatogram of propranolol (peak b, 1.5 x 10^{-5} M), with methylparaben (peak a, 1 x 10^{-4} M) as internal standard and b) typical calibration curve, using system A. For calibration curves points are the mean ± sem of at least two determinations.

a) b)

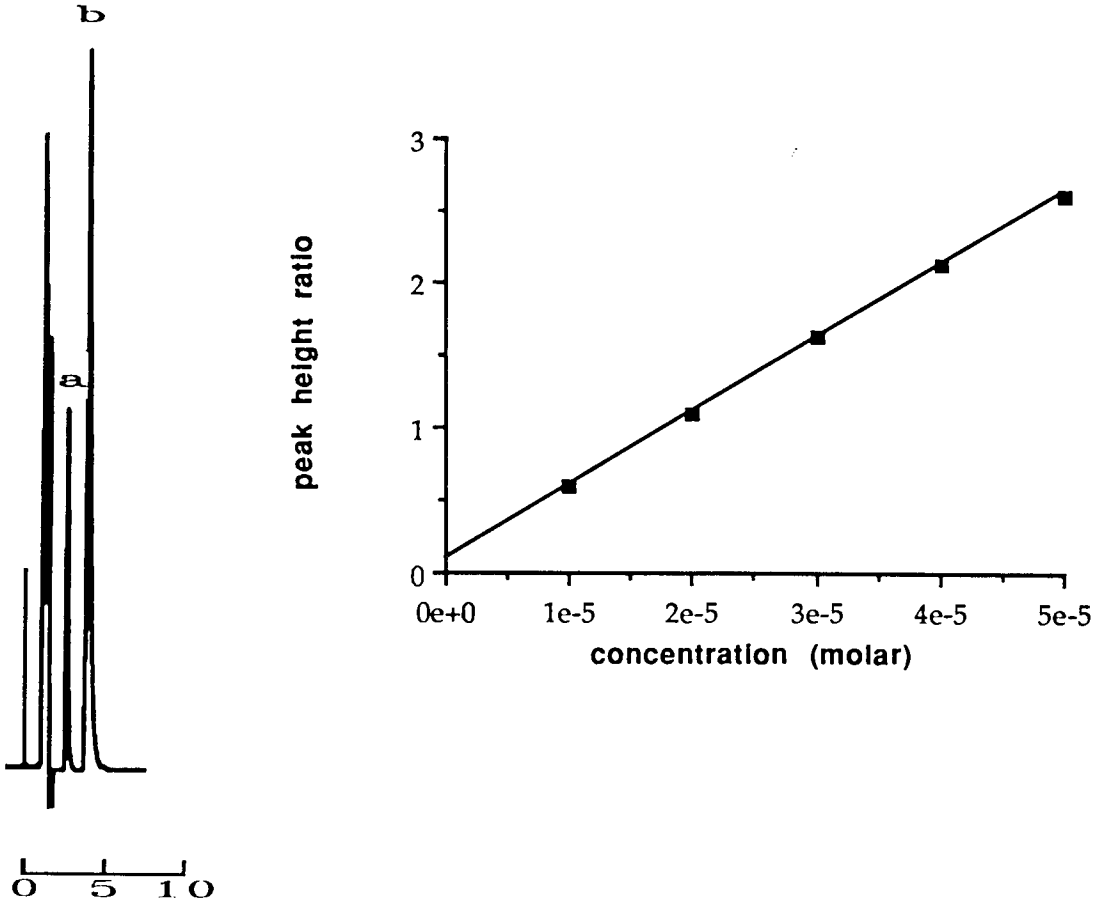


Table 2.13 Calibration statistics for propranolol (PR)

compound	slope (x 10-4)	intercept (x10²)	correlation coefficient
PR	5.084	9.586	1.000

Table 2.14 HPLC parameters for propranolol (PR) with methylparaben (MP) as internal standard. R_s is the resolution of PR from MP.

compound	t_R (min)	k'	N (m^{-1})	H (μm)	R_s
MP	3.0	2.0	23040	43.4	2.67
PR	4.0	3.0	40960	24.4	

2.3.1.6 Oxprenolol

The chemical structure of oxprenolol is given in figure 2.10

Table 2.15 HPLC conditions for oxprenolol (OX)

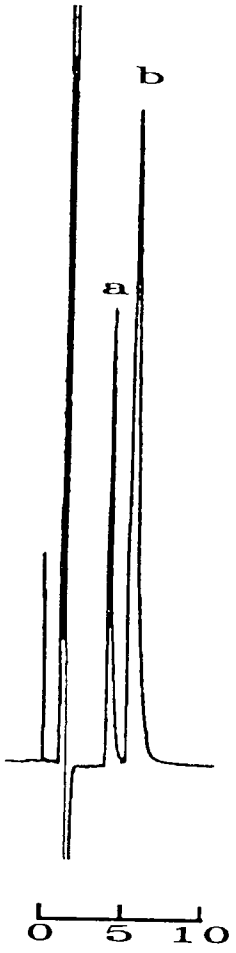
system	A
mobile phase	28% acetonitrile 0.1% diethylamine pH 2.5 with orthophosphoric acid
flow rate	1.0 mL min^{-1}
wavelength	280 nm
injection solvent	phosphate buffer (pH 7.4)
internal standard	methylparaben (1 x 10-4 M)
AUFS	0.08

Table 2.16 Calibration statistics for oxprenolol (OX)

compound	slope (x 10-4)	intercept (x10²)	correlation coefficient
OX	3.878	1.747	1.000

Figure 2.12 Examples of a) a typical chromatogram of oxprenolol (peak b, 7.5 x 10-5 M) with methylparaben (peak a, 1 x 10-4 M) as internal standard and b) typical calibration curve, using system A. For calibration curves points are the mean ± sem of at least two determinations.

a)



b)

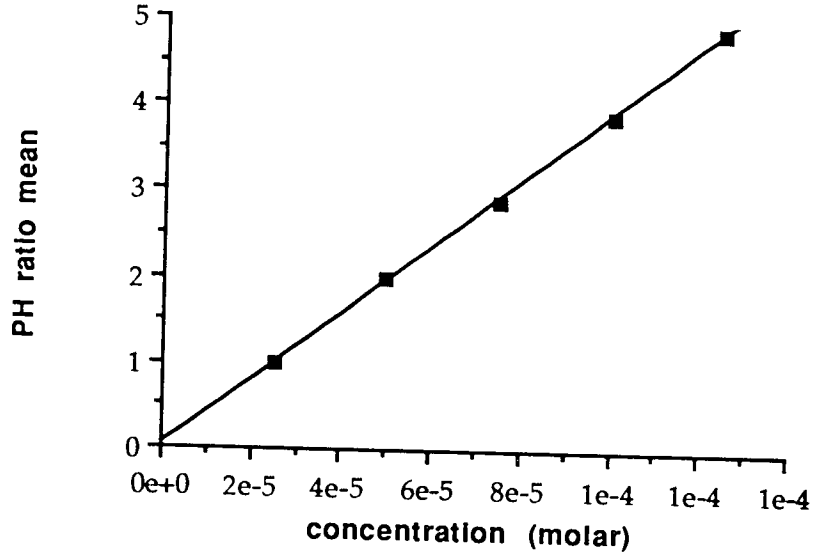


Table 2.17 HPLC parameters for oxprenolol (OX) with methylparaben (MP) as internal standard. R_s is the resolution of OX from MP.

compound	t_R (min)	k'	N (m^{-1})	H (μm)	R_s
MP	4.0	3.0	18200	54.9	2.67
OX	5.5	4.5	8600	116	

2.3.1.7 Atenolol

The chemical structure of atenolol is given in figure 2.10

Table 2.18 HPLC conditions for atenolol (AT)

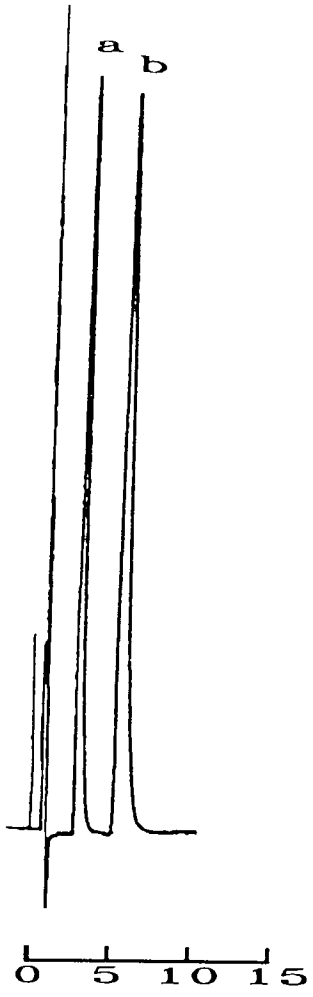
system	A
mobile phase	7.5% acetonitrile 0.1% diethylamine pH 2.5 with orthophosphoric acid
flow rate	1.0 mL min^{-1}
wavelength	280 nm
injection solvent	phosphate buffer (pH 7.4)
internal standard	TRP (5×10^{-4} M)
AUFS	0.08

Table 2.19 Calibration statistics for atenolol (AT)

compound	slope ($\times 10^{-4}$)	intercept ($\times 10^{2}$)	correlation coefficient
AT	0.6027	10.423	0.998

Figure 2.13 Examples of a) a typical chromatogram of atenolol (peak a, 1.5 x 10-4 M), with tryptophan (peak b, 5 x 10-5 M) as internal standard and b) typical calibration curve, using system A. For calibration curves points are the mean ± sem of at least two determinations.

a)



b)

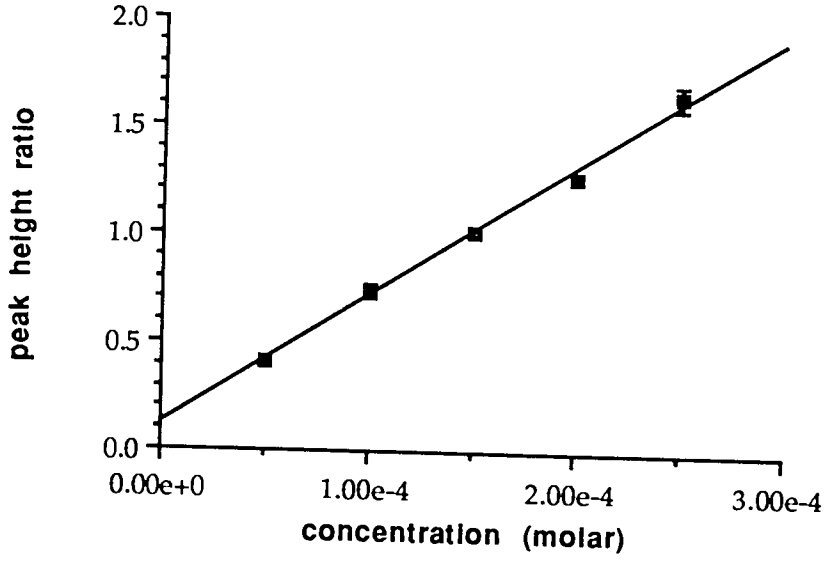


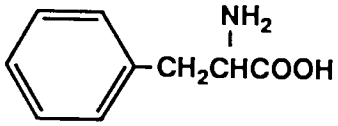
Table 2.20 HPLC parameters for atenolol (AT) with tryptophan (TRP) as internal standard. R_s is the resolution of AT from TRP.

compound	t_R (min)	k'	N (m^{-1})	H (μm)	R_s
AT	3.0	2.0	2560	390	3.14
TRP	5.75	4.75	5290	189	

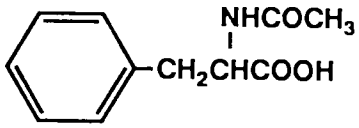
2.3.1.8 N-acetylphenylalanine

Figure 2.14 The chemical structure of a) phenylalanine, b) N-acetyl-phenylalanine, c) the methyl ester of phenylalanine and d) phenylalaninol.

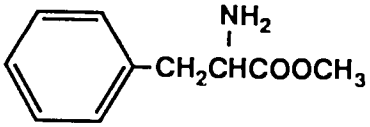
a)



b)



c)



d)

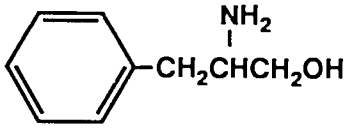
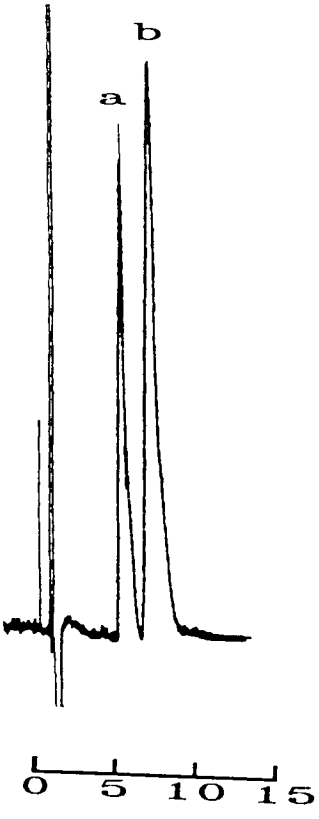


Table 2.21 HPLC conditions for N-acetylphenylalanine (NP)

system	A
mobile phase	12% acetonitrile 0.1% orthophosphoric acid
flow rate	1.0 mL min^{-1}
wavelength	255 nm
injection solvent	phosphate buffer (pH 7.4)
internal standard	0.01M phenol prepared in 10% orthophosphoric acid solution. The acidified phenol is needed to improve the peak shape. Final concentration of injected phenol 1 mM
AUFS	0.04

Figure 2.15 Examples of a) a typical chromatogram of N-acetylphenylalanine (peak b, 6 x 10-4 M), with phenol (peak a, 1 x 10-3 M) in 10% orthophosphoric acid as internal standard and b) typical calibration curve, using system A. For calibration curves points are the mean ± sem of at least two determinations.

a)



b)

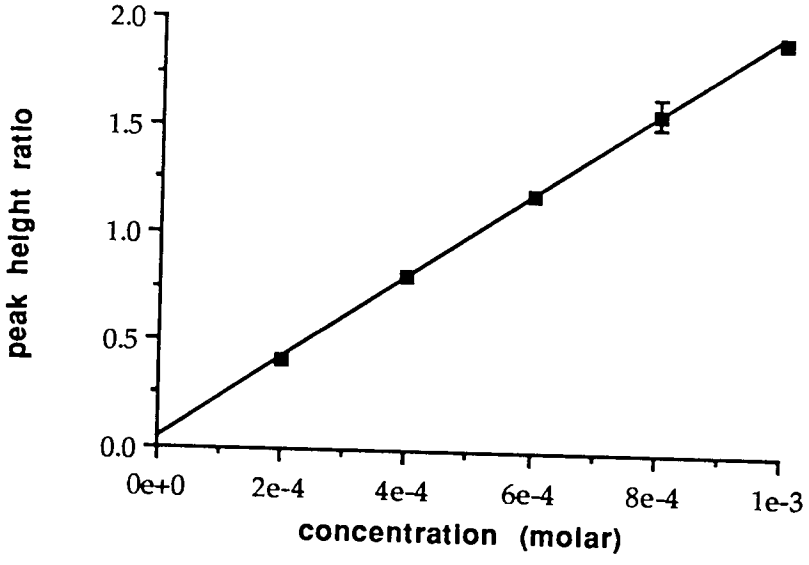


Table 2.22 Calibration statistics for N-acetylphenylalanine (NP)

compound	slope (x 10-3)	intercept (x10²)	correlation coefficient
NP	1.910	3.810	0.999

Table 2.23 HPLC parameters for N-acetylphenylalanine (NP) with phenol as internal standard. R_s is the resolution of NP from phenol.

compound	t_R (min)	k'	N (m^{-1})	H (μm)	R_s
phenol	5.5	4.5	8604	116	2.29
NP	7.5	6.5	9000	111	

2.3.1.9 The methyl ester of phenylalanine

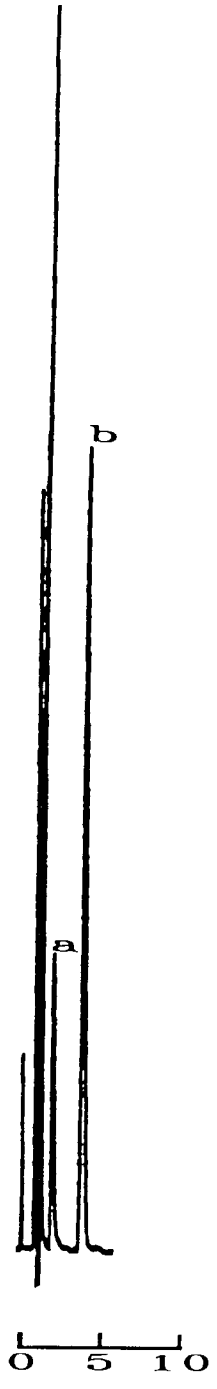
The chemical structure of the methyl ester of phenylalanine is given in figure 2.14

Table 2.24 HPLC conditions for the methyl ester of phenylalanine

system	A
mobile phase	18% acetonitrile 0.2% diethylamine pH 2.5 with orthophosphoric acid
flow rate	1.0 mL min^{-1}
wavelength	255 nm
injection solvent	phosphate buffer (pH 7.4)
internal standard	phenol (5×10^{-4})
AUFS	0.08

Figure 2.16 Examples of a) a typical chromatogram of the methyl ester of phenylalanine (peak a, 8 x 10-4 M) , with phenol (peak b, 5 x 10-4 M) as internal standard and b) typical calibration curve, using system A. For calibration curves points are the mean ± sem of at least two determinations.

a)



b)

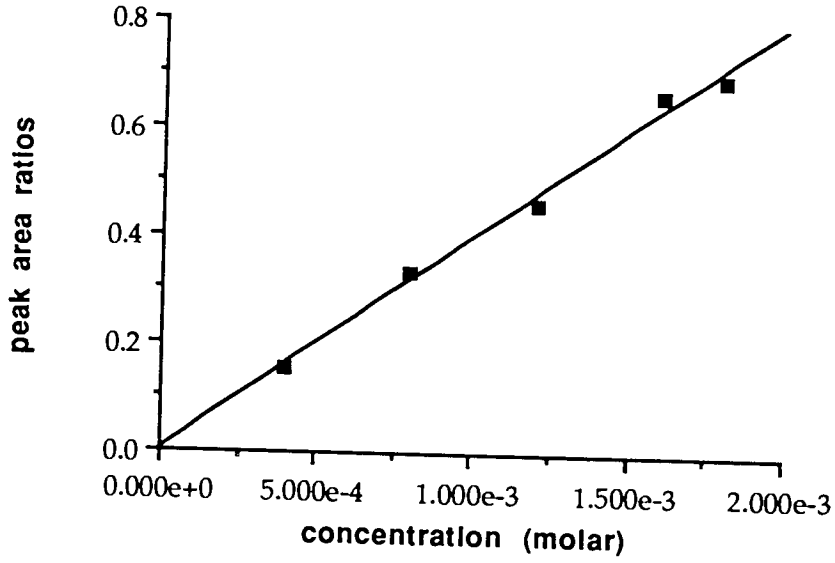


Table 2.25 Calibration statistics for the methyl ester of phenylalanine (ME)

compound	slope (x10-2)	intercept (x10³)	correlation coefficient
ME	3.9618	2.271	0.991

Table 2.26 HPLC parameters for the methyl ester of phenylalanine (ME) with phenol as internal standard. R_s is the resolution of ME from phenol.

compound	t_R (min)	k'	N (m-1)	H (µm)	R_s
ME phenol	2.0 4.0	1.0 3.0	2560 10240	390 97.7	4.00

2.3.2 HPLC methods for use with hydrolysis experiments

The development of these HPLC methods was based upon the work of Molnar and Horvath (1977), Hancock et al. (1978), Lundanes and Griebrokk (1978), Pietrzyk and Smith (1983), Wilson et al. (1981), Zweig and Sherma (1986), and Mant and Hodges (1989).

Analyses were performed using system B, where by chromatograms were collected and integrated by the chromatography workstation. The "peak response" referred to in the calibration curves for this section, are the peak areas determined by this method. The sample injection volume was 100 µL for all experiments with the exception of leucine and des-tyrosine enkephalin, where the volume was 20 µL.

2.3.2.1 Separation and detection of tryptophan, tryptophylglycine and tryptophylglycylglycine.

Table 2.27 HPLC conditions for the separation of tryptophan, tryptophylglycine and tryptophylglycylglycine

system	B
mobile phase	4% acetonitrile 0.1% diethylamine pH 3.5 with orthophosphoric acid
flow rate	1.5 mL min-1
wavelength	270 nm
injection solvent	6.67% DMSO in phosphate buffer (pH 7.4)
internal standard	none
AUFS	0.04

Table 2.28 Calibration statistics for tryptophan (TRP), tryptophylglycine (WG) and tryptophylglycylglycine (WGG)

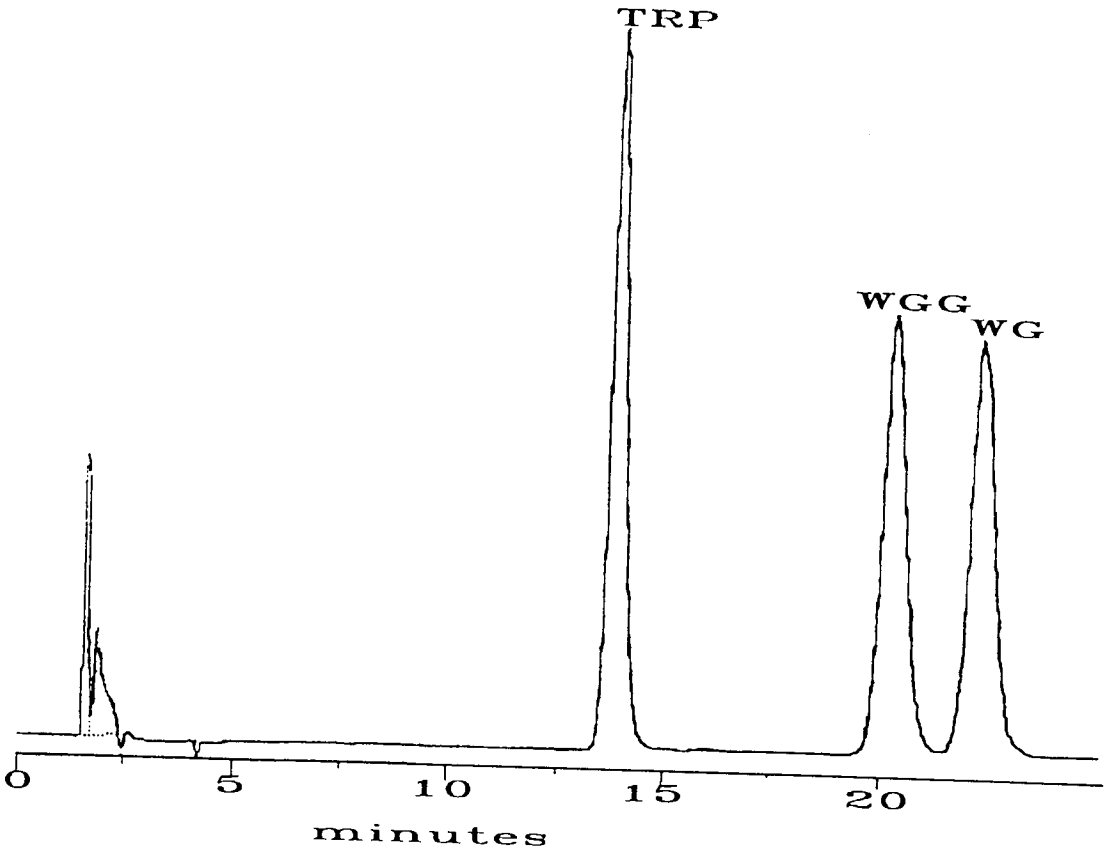
compound	slope (x10-10)	intercept (x10-4)	correlation coefficient
TRP	1.8545	-0.8718	1.000
WG	1.6695	-2.1516	0.998
WGG	1.7016	0.1016	0.998

Table 2.29 HPLC parameters for tryptophan (TRP), tryptophylglycine (WG) and tryptophylglycylglycine (WGG) with no internal standard. R_s is the resolution of WGG from TRP and WG from WGG.

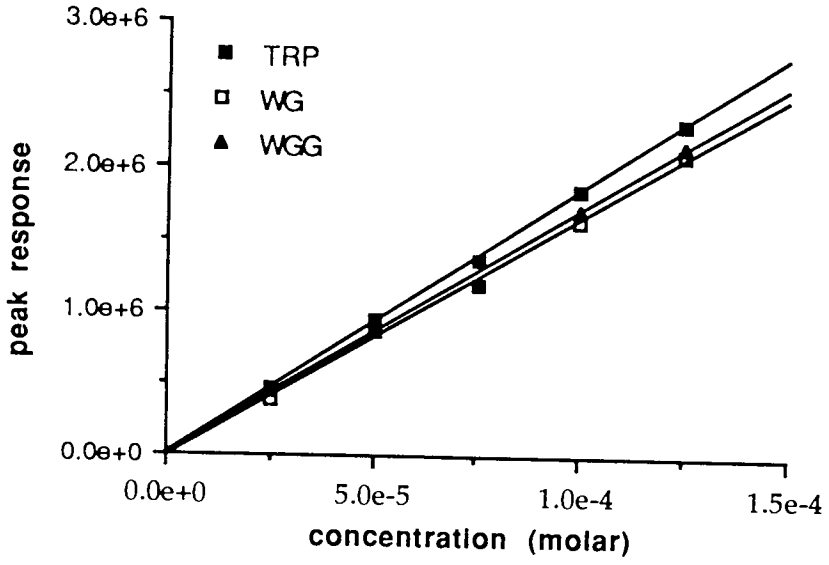
compound	t_R (min)	k'	N (m-1)	H (μm)	R_s
TRP	13.79	7.73	54091	18.5	7.36
WGG	20.23	11.80	65480	15.27	1.86
WG	22.21	13.06	62361	16.04	

Figure 2.17 Examples of a) a typical chromatogram of tryptophan, tryptophylglycine and tryptophylglycylglycine without an internal standard, each at a concentration of 1.25 x 10^{-4} M and b) typical calibration curve, using system B. For calibration curves points are the mean ± sem of at least two determinations.

a)



b)



2.3.2.2 The separation of phenylalanine, phenylalanylglycine, phenylalanylglycylglycine, glycylphenylalanine and glycylglycyl-phenylalanine

Table 2.30 HPLC conditions for the separation of phenylalanine, phenylalanylglycine, phenylalanylglycylglycine, glycyl-phenylalanine and glycylglycylphenylalanine.

system	B
mobile phase	3% acetonitrile 0.1% diethylamine pH 3.5 with orthophosphoric acid
flow rate	1.0 mL min^{-1}
wavelength	255 nm
injection solvent	6.67% DMSO in phosphate buffer (pH 7.4)
internal standard	none
AUFS	0.04

Figure 2.18 The chemical structures of phenylalanine (PHE), phenylalanylglycine (FG), phenylalanylglycylglycine (FGG), glycylphenylalanine (GF) and glycylglycyl-phenylalanine (GGF)

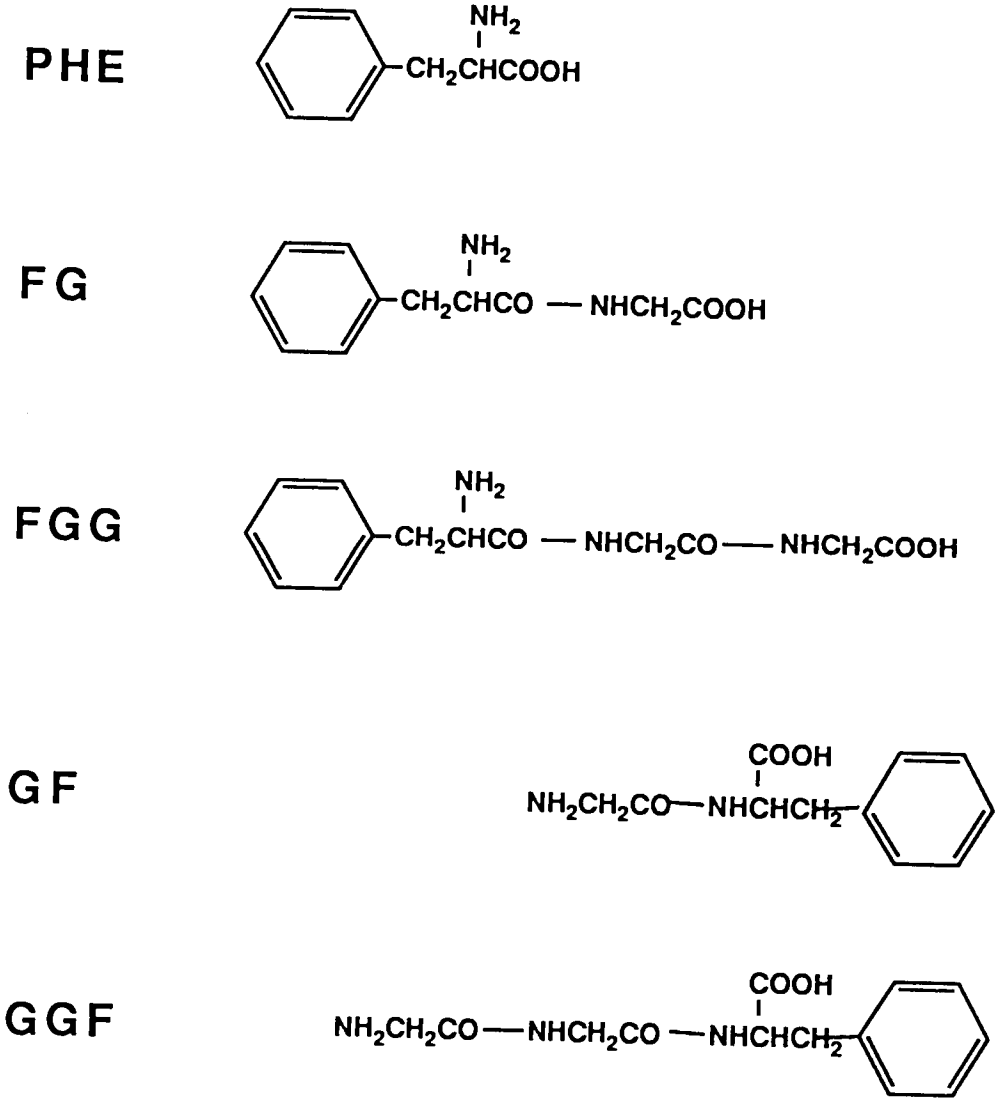
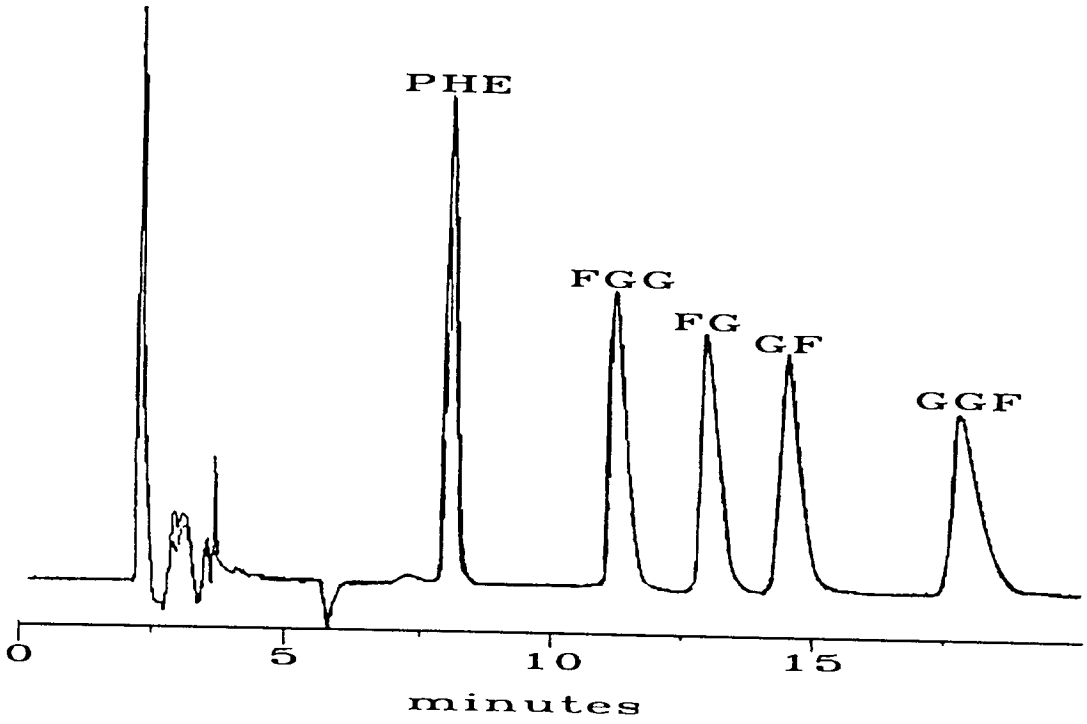
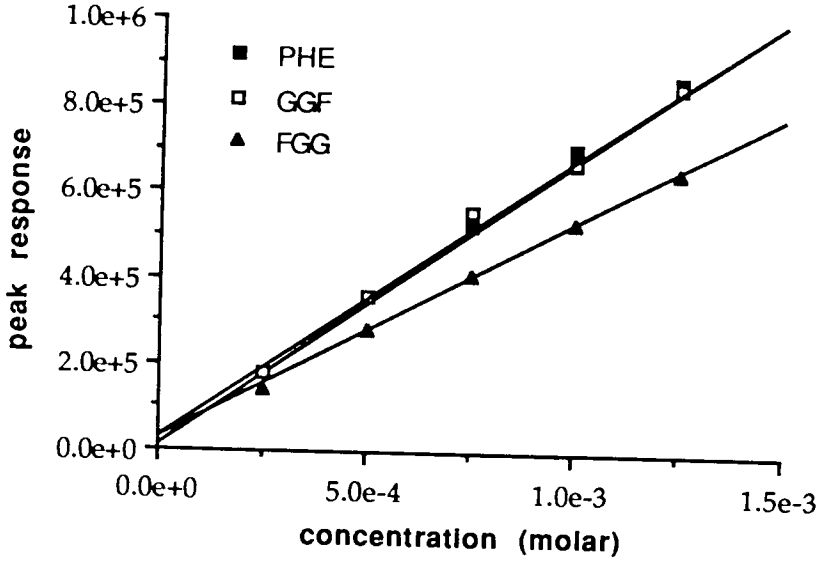


Figure 2.19 Examples of a) a typical chromatogram of phenylalanine (PHE), phenylalanylglycine (FG), phenylalanylglycylglycine (FGG), glycylphenylalanine (GF) and glycylglycylphenylalanine (GGF), each at a concentration of 1 x 10^{-3} M, without an internal standard and b) typical calibration curves of PHE, GGF and FGG and c) GF and FG, using system B. For calibration curves points are the mean ± sem of at least two determinations.

a)



b)


1.0e+6
8.0e+5
6.0e+5
4.0e+5
2.0e+5
0.0e+0
peak response
PHE
GGF
FGG
0.0e+0
5.0e-4
1.0e-3
1.5e-3
concentration (molar)

c)

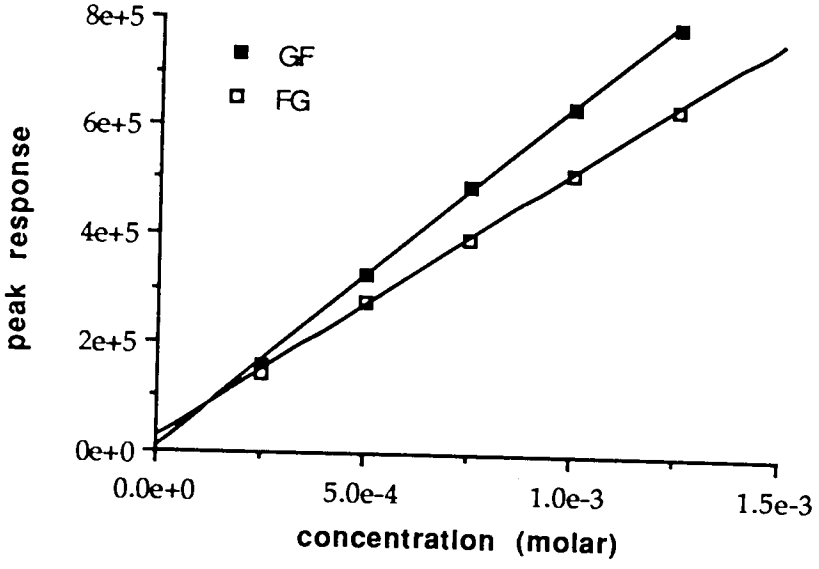

8e+5
6e+5
4e+5
2e+5
0e+0
peak response
GF
FG
0.0e+0
5.0e-4
1.0e-3
1.5e-3
concentration (molar)

Table 2.31 Calibration statistics for figure 2.19b and c

compound	slope (x10-8)	intercept (x10-4)	correlation coefficient
PHE	6.8811	0.9060	0.999
FG	4.9620	2.3393	0.999
FGG	5.0707	2.3387	0.999
GF	6.3563	0.5603	0.999
GGF	6.6524	2.4674	0.997

Table 2.32 HPLC parameters for of phenylalanine (PHE), phenylalanylglycine (FG), phenylalanylglycyl-glycine (FGG), glycylphenylalanine (GF) and glycylglycylphenylalanine (GGF). R_s is the resolution of consecutive peaks on the chromatogram from each other.

compound	t_R (min)	k'	N (m-1)	H (μm)	R_s
PHE	8.1	1.53	65610	15.3	6.60
FGG	11.4	2.56	57760	17.3	2.67
FG	13.0	3.06	75110	13.3	2.30
GF	14.5	3.53	68653	14.57	3.88
GGF	17.8	4.56	50694	19.73	

2.3.2.3 Phenylalanylglycylglycylphenylalanine and its metabolites

To determine the concentration of phenylalanylglycylglycylphenylalanine (FGGF) an isocratic methodology was developed, which is described in table 2.33. This was developed after unsuccessful resolution of FGGF by gradient elution, from excessive baseline noise in the latter stages of the chromatogram. The concentration of the metabolites of FGGF were determined using the conditions described in table 2.34, which comprises an initial isocratic method followed by a gradient to elute the parent compound. The conditions for the initial isocratic period are the same as described in table 2.30 and possesses the same peak retention times and HPLC parameters as described in table 2.32.

Figure 2.20 The chemical structure of phenylalanylglycylglycyl-phenylalanine

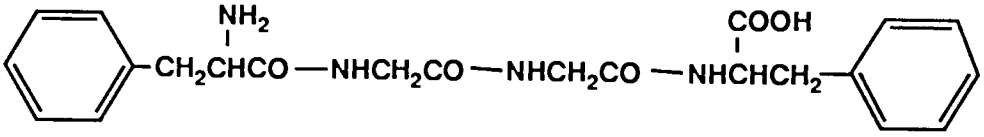


Table 2.33 HPLC conditions for isocratic determination of phenylalanylglycylglycylphenylalanine

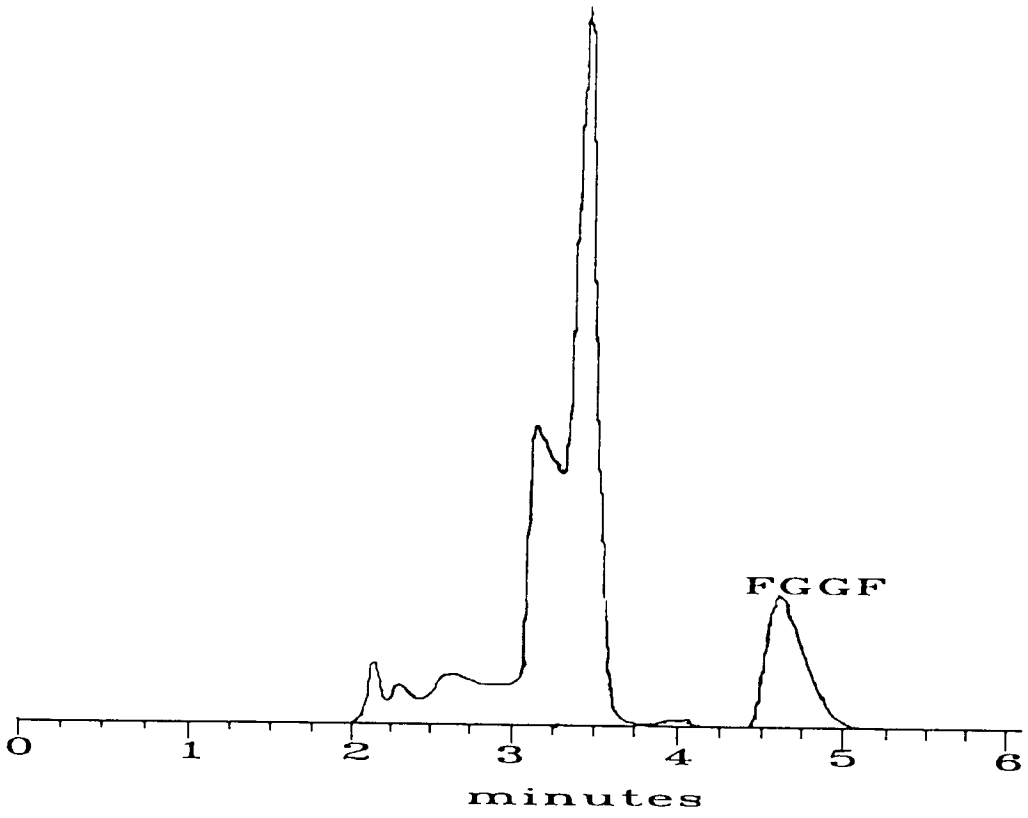
system	B
mobile phase	acetonitrile : 0.1% trifluoroacetic in HPLC water, 30:70
flow rate	1.0 mL min^{-1}
wavelength	255 nm
injection solvent	6.67% DMSO in phosphate buffer (pH 7.4)
internal standard	none
AUFS	0.04

Table 2.34 Gradient program to elute phenylalanylglycylglycyl-phenylalanine from the column after determination of its metabolites. Where B comprises 3% acetonitrile, 0.1% diethylamine adjusted to pH 3.5 and C is 100% acetonitrile. Curve 6 is the gradient program which produces a linear change in %C with time, rather than a concave or convex profile.

time	%B	%C	curve
0	100	0	
17	100	0	
22	5	95	6
27	5	95	
30	100	0	6
35	100	0	

Figure 2.21 Examples of a) a typical isocratic chromatogram of phenylalanylglycylglycylphenylalanine (FGGF) (8 x 10-4 M) without an internal standard and b) typical calibration curve, using system B. For calibration curves points are the mean ± sem of at least two determinations.

a)



b)

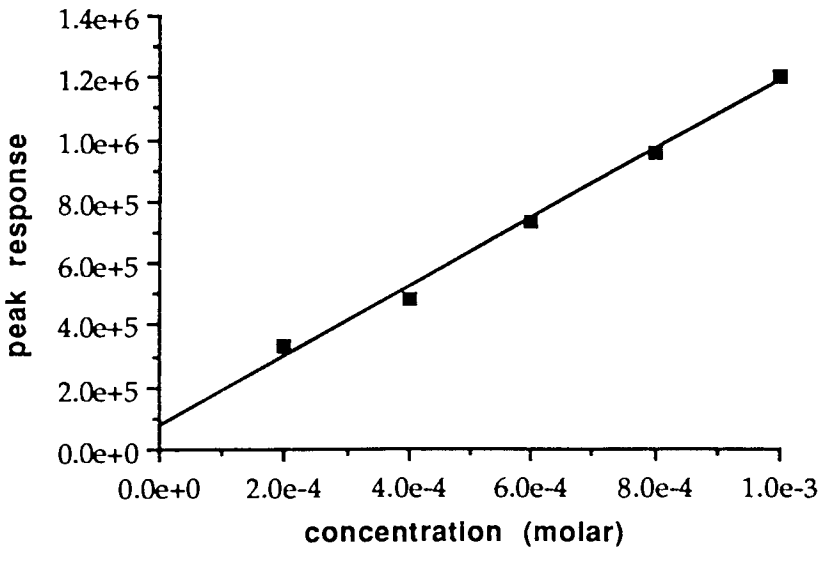


Table 2.35 Calibration statistics for phenylalanylglycylglycyl-phenylalanine (FGGF).

compound	slope (x10-9)	intercept (x10-4)	correlation coefficient
FGGF	1.1183	7.5284	0.997

Table 2.36 HPLC parameters for phenylalanylglycylglycyl-phenylalanine (FGGF) with no internal standard.

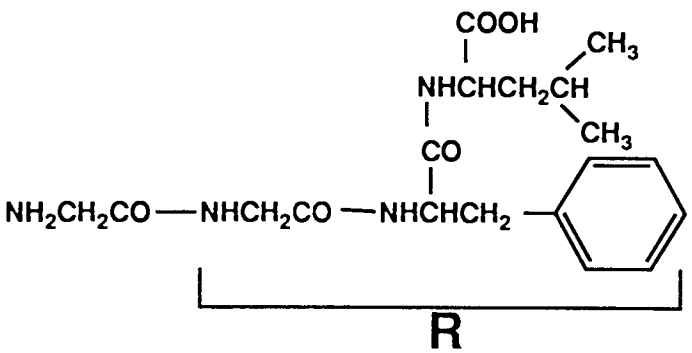
compound	t_R (min)	k'	N (m-1)	H (μm)
FGGF	4.6	1.05	19090	52.4

2.3.2.4 Leucine enkephalin and des-tyrosine leucine enkephalin

The determination of leucine enkephalin (YGGFL) and des-tyrosine leucine enkephalin (GGFL) employs the method of Hussain et al. (1990).

Figure 2.22 The chemical structures of a) des-tyrosine leucine enkephalin and b) leucine enkephalin.

a)



b)

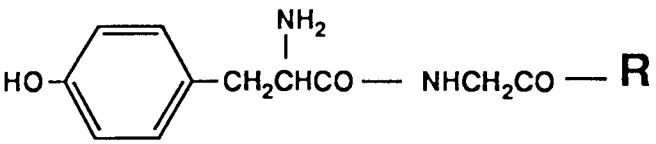


Table 2.37 HPLC conditions for the separation of leucine enkephalin and des-tyrosine leucine enkephalin.

system	B
mobile phase	acetonitrile : 0.05M K_2HPO_4, 30:70 pH 3.0 with orthophosphoric acid
flow rate	0.8 mL min^{-1}
wavelength	205 nm
injection solvent	phosphate buffer (pH 7.4)
internal standard	none
AUFS	0.04

Table 2.38 Calibration statistics for leucine enkephalin (YGGFL) and des-tyrosine leucine enkephalin (GGFL)

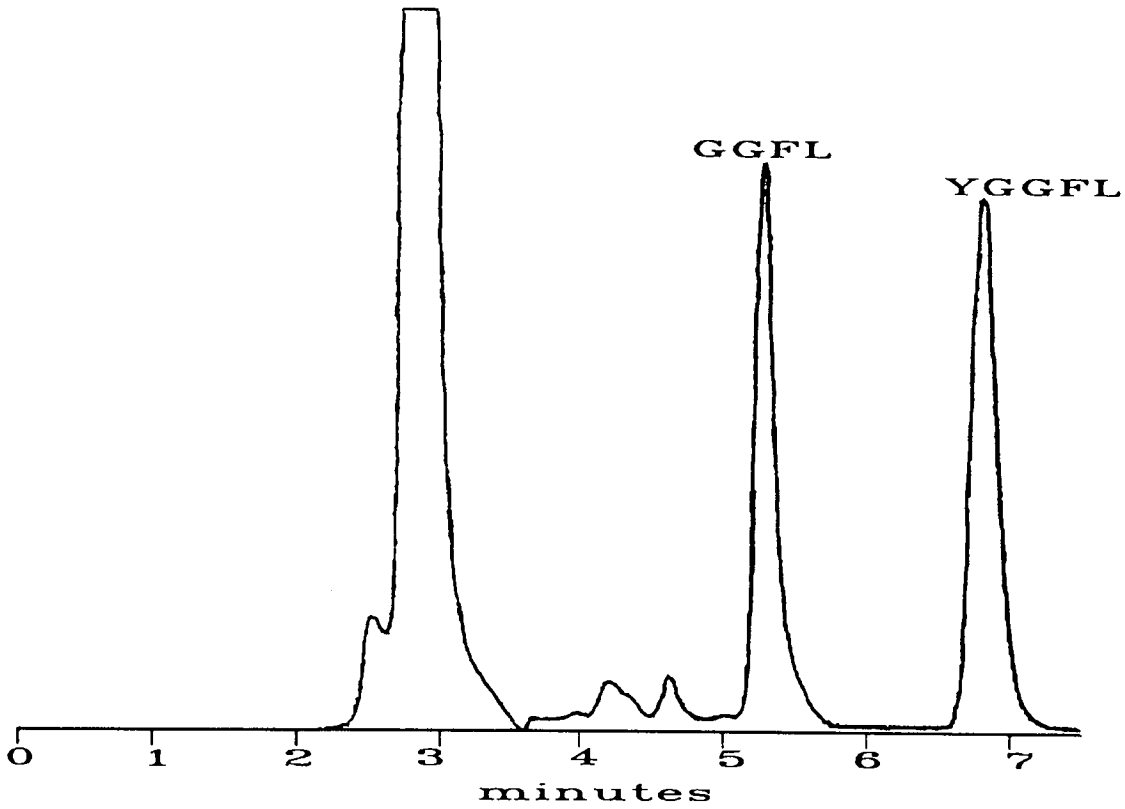
compound	slope ($x10^{-10}$)	intercept ($x10^{-4}$)	correlation coefficient
YGGFL	4.1865	-2.5069	1.000
GGFL	3.4474	-1.4060	0.999

Table 2.39 HPLC parameters for leucine enkephalin (YGGFL) and des-tyrosine leucine enkephalin (GGFL) without an internal standard. R_s is the resolution of GGFL from YGGFL.

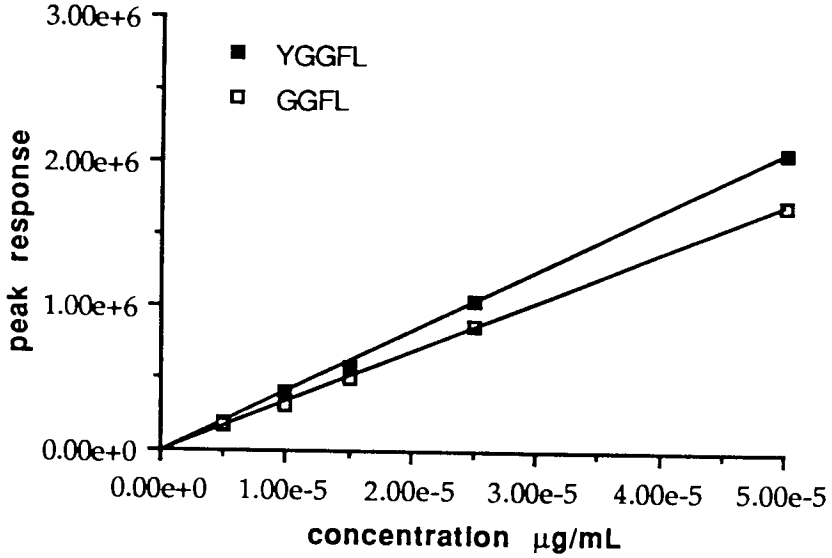
compound	t_R (min)	k'	N (m^{-1})	H (μm)	R_s
GGFL	5.3	0.8	87776	11.4	5.40
YGGFL	6.8	1.4	64761	15.4	

Figure 2.23 Examples of a) a typical chromatogram of leucine enkephalin (YGGFL) and des-tyrosine leucine enkephalin (GGFL), each at a concentration of 25 μg mL-1 without an internal standard and b) typical calibration curve, using system B. For calibration curves points are the mean ± sem of at least two determinations.

a)



b)


3.00e+6
2.00e+6
1.00e+6
0.00e+0
peak response
YGGFL
GGFL
0.00e+0
1.00e-5
2.00e-5
3.00e-5
4.00e-5
5.00e-5
concentration µg/mL

CHAPTER 3
MUCUS PREPARATION

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MUCUS PREPARATION

3.1 INTRODUCTION

In a normal, healthy human the quantity of nasal secretion is scarce being typically 5 μm thick (Hilding et al., 1973). It is therefore difficult to obtain large quantities that are representative of mucus *in vivo*. Larger quantities of nasal secretions are produced in a variety of disease states including allergy, common cold and in those suffering from the genetic disorder cystic fibrosis, but they are not normal and are probably contaminated with inflammatory products and bacteria (Melon, 1969; Tremble, 1949; Hilding et al., 1973; Lorin et al., 1976; Marom et al., 1984).

A variety of techniques have been employed to collect nasal secretions for analysis including nasal suction (Hilding, 1972), aspiration (Majima et al., 1988) and nasal wash techniques (Powell et al., 1977). An alternative approach has been to collect the secretions onto absorbents such as filter paper (Lorin et al., 1972) and cellulose sponges (Wilson and Allansmith, 1976) introduced into the nose. A few workers have used nasal provocation tests with methacholine, histamine, atropine sulphate and allergens to evoke secretion (Brofeldt et al., 1986; Pelikan and Pelikan-Filipek, 1988; Raphael et al., 1988). Finally, there is an interest in using cultured nasal cells as a source of secretions for subsequent analysis (Boat et al., 1974; Patow et al., 1984a and b).

There are disadvantages associated with each of the collection methods outlined above in addition to the small yield. The material obtained from nasal washes has been diluted by an unknown volume of wash fluid and is invariably contaminated with secretions of lachrymal origin (Patow et al., 1984a). The composition of secretions collected on absorbents is very dependent on the careful placement and removal of the absorbent from the nasal cavity. This affects the protein concentration (Eichner, 1983) and the fluid may also be contaminated by tears, cell debris (if abrasion of the mucosa has occurred) and

serum components due to increased transudation (Patow et al., 1984b). Remington et al. (1964) have reported that secretions produced by this method are abnormal.

Nasal provocation produces the largest volumes of secretion and this has been performed in order to investigate the composition of nasal secretions with respect to allergy and rhinitis. The biochemical composition of the secretion was found to depend on the chemical agent employed and, in the case of histamine, evoked marked transudation (Brofeldt et al., 1986). The cell culture techniques appear very promising for producing uncontaminated nasal secretions but it still may be argued that there is no certainty that they are representative of nasal secretions *in vivo*. There are also difficulties in culturing differentiated cell layers.

Therefore, in this study, pig gastric mucus was chosen as a model to study the role of mucus as a barrier to peptide delivery. Unlike human nasal secretions, pig gastric mucus is readily available in large quantities and its composition and structure have been extensively studied (Allen, 1978; Allen et al., 1981; Hutton et al., 1988; Robertson et al., 1989). The barrier properties have been investigated with crude native mucus scraped from the gastric or intestinal mucosa at one extreme (Nimmerfall and Rosenthaler, 1980; Turner et al., 1985; Desai and Vadgama, 1991) and purified glycoprotein gels at the other (Hughes, 1988).

Crude gastric mucus is susceptible to degradation by proteolytic enzymes present in the gastrointestinal tract. For example, trypsin and pepsin, have been shown to reduce the molecular weight of the glycoprotein by 3 %, by removing part of the protein core (Scawen and Allen, 1977). Proteolysis by pepsin and other enzymes has resulted in solubilisation of the mucus gel, with a loss of its characteristic elasticity and gel-forming properties (Bell et al., 1985). Therefore it seems necessary to minimise the degradation of the gel with the use of appropriate enzyme inhibitors in order to maintain its structure and nature.

Purified glycoprotein gels are obtained by a 2-stage process of initial solubilisation (e.g. proteolysis, disulphide-bond reduction or mechanical shear) followed by separation of the mucins from the other components, typically by gel-exclusion chromatography or caesium chloride density gradient ultracentrifugation (Lethem, 1987). The purified glycoproteins are then reconstituted in buffer and have been found to gel at the same concentration of glycoprotein as that present in the gel *in vivo* (Brown et al., 1981b; Mantle and Allen, 1981a). The disadvantage with this methodology is the large quantity of mucus required and the time taken in purification. Perhaps of greater importance is the fact that other components within the mucus gel *in vivo*, which are removed by this process, may play a role in retarding peptide and protein molecules.

Non-glycoprotein components within the mucus gel including proteins and lipids are postulated by some workers to play a role in creating the viscosity and barrier nature of the gel (List et al., 1978; Marriott et al., 1981; Readman, 1983; Murty et al., 1984) although the protein effect has been disputed (Mantle and Allen, 1981a). Removal of the associated lipids from dog gastric mucus has been observed to increase the permeability of the gel to hydrogen ions (Sarosiek et al., 1984).

It was decided in these studies to investigate the barrier nature of the whole pig gastric mucus secretion to peptide drug delivery. The following method of mucus treatment and storage was developed and evaluated as a means of obtaining sufficient quantities of mucus that were representative of the gel *in vivo*. This was determined on the basis of water, protein and hexose content and rheological properties, all of which are established characteristics of mucus gels.

3.2 THE RHEOLOGY OF MUCUS AND THEORETICAL TREATMENT OF RESULTS

Rheology is the science of the flow and deformation of matter with the most straight forward rheological behaviour being exhibited by Hookean solids and Newtonian fluids. A Hookean, elastic solid when stressed, deforms immediately by an amount proportional to the applied stress

and maintains a constant deformation as long as the stress remains constant. Upon removal of the stress the solid regains its original shape. This elastic deformation is described by Hooke's law:

$$\tau = G.\gamma \qquad (3.1)$$

where τ is the applied stress, G is elasticity (shear modulus) and γ is strain. The elasticity and stress have the same units, Nm^{-2}, since strain is non-dimensional (Young, 1976)

If stress is applied to a Newtonian fluid it deforms by an amount proportional to the stress as defined by Newtons law, which states:

$$\tau = \eta.\dot{\gamma} \qquad (3.2)$$

where τ is the applied stress, η is viscosity ($Nm^{-2}s$) and $\dot{\gamma}$ is the rate of strain (s^{-1}). A Newtonian fluid shows no recovery when the stress is removed, as the energy is dissipated as heat in overcoming the internal friction and permanent deformation occurs.

Many materials exhibit a mechanical behaviour intermediate between these two extremes and possess both viscous and elastic properties. Consequently, they are classified as being viscoelastic. When viscoelastic materials are stressed, some of the energy involved is stored elastically and the remainder is dissipated as heat.

Mucus is one example of a viscoelastic material, exhibiting different properties depending upon the magnitude of the stress applied and the time duration of the stress. It will, however, only behave as a true viscoelastic material at very small strains. If a small stress is applied for a short time the mucus will exhibit elastic properties, but it may flow and exhibit viscous properties if the stress is applied for a greater length of time. The viscous behaviour observed at higher rates of shear will not be ideal Newtonian.

Several methods have been applied to determine the rheological properties of mucus preparations (Marriott, 1981). Some of these methods can be classed as viscometric techniques. These include the U-tube viscometer used by Brown et al. (1981a) and rotational viscometers, which have been used by several workers (List et al., 1978; Mantle and Allen, 1981a; Murty et al., 1984). The main disadvantage of the viscometric techniques is that they permit only the measurement of viscosity.

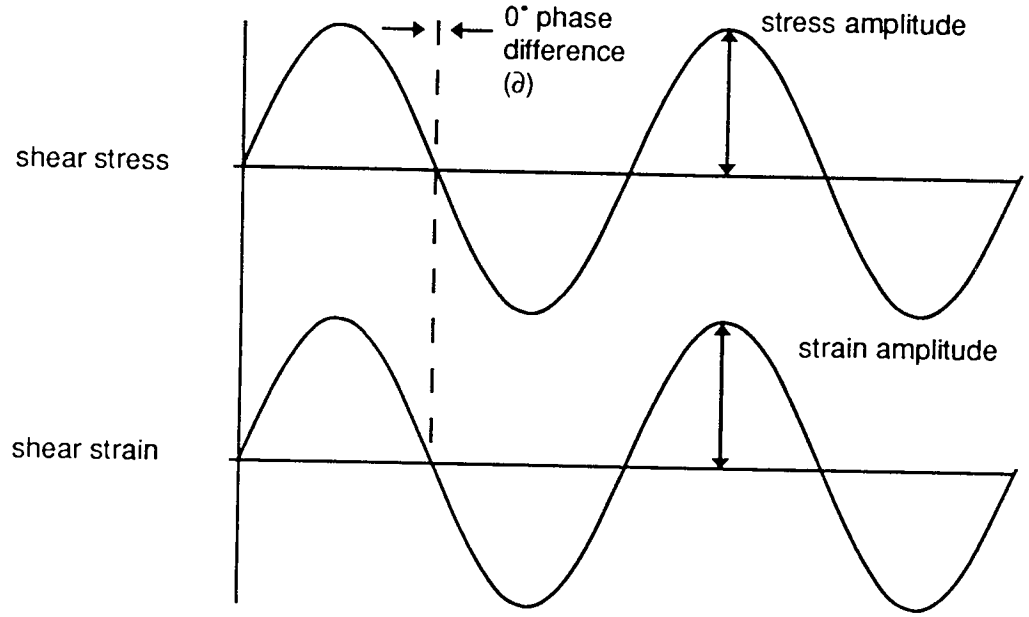
The viscous and elastic components of mucus are both important for successful clearance of respiratory mucus by cilia and therefore both properties need to be assessed when determining the physical nature of mucus. Two different types of test are available to determine viscosity and elasticity simultaneously, the static or creep compliance test, where a small, constant force is applied to the sample and the resultant strain is measured (Brown et al., 1981a; Lethem, 1987; Martin et al., 1990) and the dynamic or oscillatory test, where a sinusoidally varying stress is applied and the resultant strain recorded (Litt et al., 1976; Bell et al., 1985; Kerr et al., 1990 and Livingstone et al., 1990).

In this study dynamic oscillatory rheological evaluation was performed with a Carri-med rheometer. In this oscillatory test a sinusoidally oscillating stress is applied to the sample and the resultant sinusoidally varying displacement measured and compared to the stress. Therefore, if one cycle is considered to be a sine wave covering 360°, it is possible to compare the phase difference, δ, between the applied stress and the resultant strain wave-forms. As already stated, for an ideal, elastic solid the strain is proportional to the stress as stated in equation 3.1, this means that the strain response wave-form will be in-phase with the stress wave-form as shown in figure 3.1a.

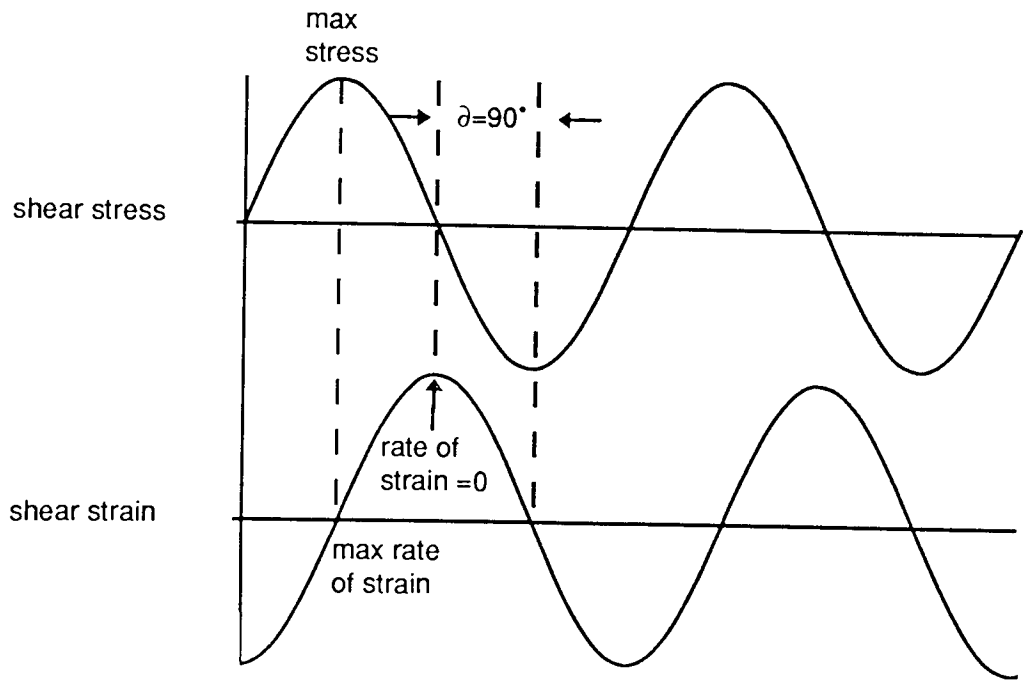
For an ideal, viscous fluid the rate of shear is proportional to the applied stress, as stated in equation 3.2, which means that the stress and the strain wave-forms are 90° out of phase, as illustrated in figure 3.1b. The reason for this is that when stress is at a maximum,

Figure 3.1 A diagram to illustrate the relationship between the wave-forms of an applied sinusoidally oscillating stress and the resulting strain for a) an ideal Hookean solid and b) an ideal Newtonian fluid.

a)



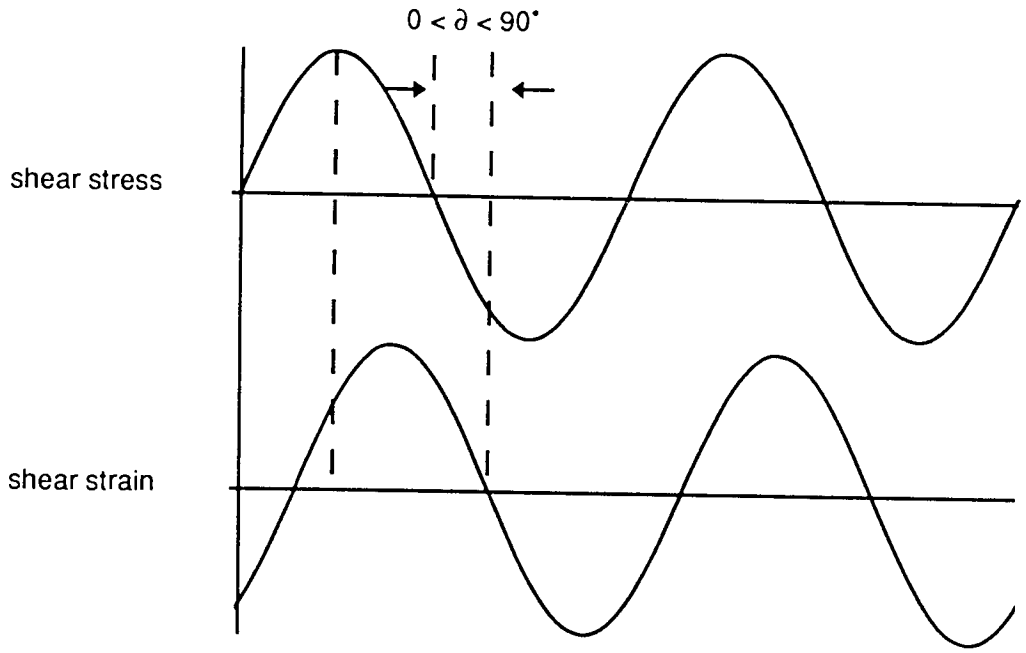
b)



strain rate is at a maximum, and at this point the strain wave-form goes through zero. Similarly, when the strain wave-form is at a maximum, the rate of change of strain is zero and stress will have a value of zero.

Viscoelastic materials will exhibit strain wave-forms within the ranges for the perfect solid and fluid, i.e. the phase angle will vary between 0 and 90°. This is illustrated in figure 3.2. The value of δ will depend upon the nature of the material, with a larger δ value indicating a more fluid-like response.

Figure 3.2 A diagram to illustrate the relationship between the wave-forms of an applied sinusoidally oscillating stress and the resulting strain for a viscoelastic material.



In addition to measuring δ, if the amplitudes of the stress and the resulting strain are recorded, the ratio to each other, G*, provides the complex modulus. The overall response G* can then be resolved into the elastic component, G', called the storage modulus and the viscous

component, G'', the loss modulus by classical mathematical techniques and trigonometry.

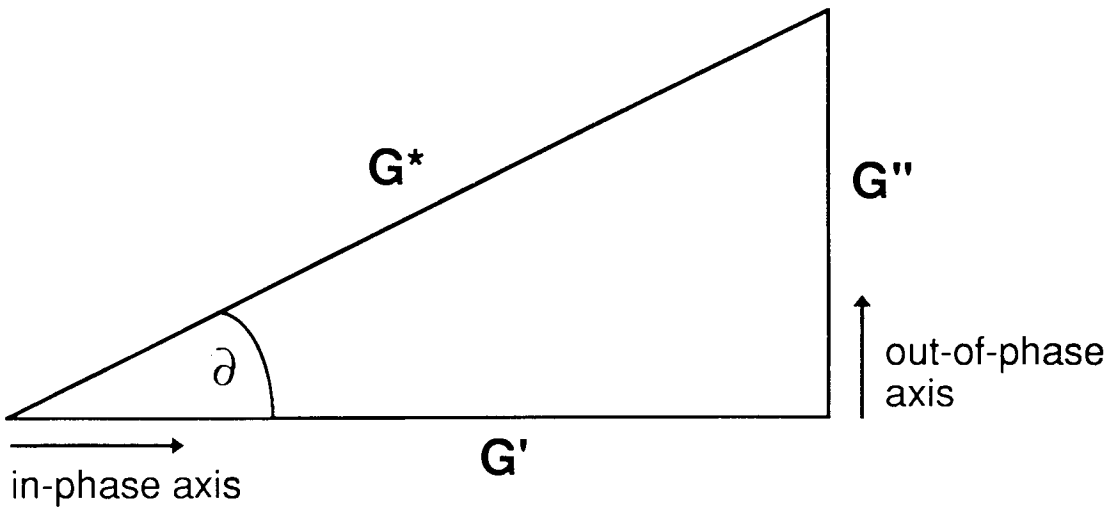
G* can be considered a complex number comprising a real (in-phase) component G' and an imaginary (out-of-phase) part G'' according to the following equation:

$$G^* = G' + i.\ G'' \qquad (6.3)$$

where i is the square root of minus one.

The different values can be resolved using trigonometry as illustrated in figure 3.3

Figure 3.3 A diagram to illustrate the trigonometric resolution of G' and G'' from G*.



Using this diagram it is clear that G' and G'' can be expressed by equations 6.4 and 6.5.

$$G' = G^*\cos\delta \qquad (6.4)$$

$$G'' = G^*\sin\delta \qquad (6.5)$$

Software integrated within the rheometer automatically performed these calculations, after the sample run was complete.

3.3 EXPERIMENTAL

3.3.1 General method of mucus treatment

The stomachs of (typically four) freshly slaughtered pigs (*Suis scrofa domestica*) were obtained from a local abattoir when required and returned to the laboratory on ice. Each stomach was opened along the lesser curvature with scissors, the food contents removed and the tissue rinsed gently with distilled water. The mucus was collected by gently scraping the surface with a plastic spatula, taking care not to remove the underlying mucosa.

The mucus from all of the stomachs was pooled, then mixed for 30 min with an equal volume of a protease inhibitor solution. This solution comprised 0.9 % w/v sodium chloride, 0.02 % w/v sodium azide, 5 mM EDTA and 1 mM phenylmethylsulphonylfluoride (PMSF) (Lethem, 1987; Livingstone, 1990). This was added to prevent enzymic degradation of the glycoprotein molecules by serine proteases and metalloproteases. The diluted mucus was then transferred into a dialysis membrane (Medicel) with a molecular weight cut off of 10,000, and dialysed overnight against ice-cold, stirred protease inhibitor solution, which was changed several times.

The diluted mucus was reconcentrated to its original volume by either centrifugation or ultrafiltration. Centrifugation was performed at 20,000 *g* for two x 30 min periods at 4°C in a Beckman J221 centrifuge. This was found to be the minimum time needed to reduce the mucus to the original volume. Ultrafiltration was performed with an Amicon model 8200, stirred ultrafiltration cell, which had a capacity of 200 mL. The membranes employed were Diaflo PM10 membrane (molecular weight cut off 10,000). The mucus was ultrafiltrated until the mucus was reduced to the initial volume, which was found typically to be overnight.

The mucus was then evaluated by biochemical and rheological means.

3.3.2 Biochemical characterisation

3.3.2.1 Dry weight determination

A known mass of mucus (0.1 g) was weighed onto a pre-weighed piece of aluminium foil and the initial mass of the mucus, M_I, recorded. The mucus sample was then dried in an oven at 100°C until all of the water had been removed and the sample had attained a constant weight, M_D. The dry weight was then determined as a percentage of the initial weight. Experiments were performed in triplicate for each batch of mucus.

3.3.2.2 Protein determination

The protein determined in this assay will be a total of protein associated with the mucus gel (due to proteins such as albumin and immunoglobulins) and additionally protein comprising the mucus glycoprotein molecules. Cells damaged during scraping the mucus from the stomach epithelia may release protein into the mucus secretion, which will also be measured by this analysis (Bell et al., 1985).

The concentration of protein was determined by the protein-dye binding method (Bradford, 1976). The basis of the assay is the formation of a complex between the acidified, anionic dye molecules and the NH_3^+ groups of the sample protein (Fazekas et al., 1963). The formation of the stable, blue coloured complex produces a shift in absorbance from 465-595 nm. The binding of the dye appears to be independent of the molecular weight of the protein above 3000 Da, below this value no binding occurs (Sedmak and Grossberg, 1977). Therefore, by constructing a standard curve of known protein concentration versus absorbance at 595 nm the protein concentration in the unknown samples can be determined.

The analytical reagent was prepared as follows. Coomassie brilliant blue G-250 (100 mg) was dissolved in 50 mL 95% ethanol. To this solution 100 mL 85% w/v phosphoric acid was added. The resulting solution was diluted up to one litre with distilled water. This reagent was stored at 4°C and used within 3 months of preparation.

A stock solution containing 100 µg of bovine serum albumin (BSA) in 0.1 mL was prepared in the protease inhibitor solution (section 3.3.1). Dilutions of this solution were made to produce standards containing 20, 40, 60 and 80 µg in 0.1 mL. A sample (0.1 mL) of each standard was pipetted in to a test tube followed by 5 mL analytical reagent and the contents were mixed by vortexing. The samples were incubated at room temperature for 2 min then the absorbance read at 595 nm within 1 hr., against a reagent blank prepared from 0.1 mL of the protease inhibitor solution and 5 mL of protein reagent. Each standard was determined in duplicate.

A sample of mucus (0.05 g) was weighed accurately in to a test tube. A sample (5 mL) of protease inhibitor solution as described in section 3.3.1 was then added and the samples were mixed to disperse the mucus. Samples (0.1 mL) of the suspension were transferred to separate test tubes and 5 mL of the protein reagent was added. The mixture was shaken, then incubated at room temperature for 2 min. After this time the absorbance at 595 nm was determined with the protease inhibitor solution employed as the reference solution. Experiments were performed in triplicate for each batch of mucus tested. The protein concentration was determined by interpolation from the linear calibration curve obtained by linear regression.

3.3.2.3 Hexose determination

The hexose content was determined using a slightly modified orcinol method for protein-bound hexose (Winzler, 1955) as described by Walters (1986).

A sulphuric acid solution (solution A) was prepared with 10 mL distilled water and 90 mL conc. sulphuric acid. The orcinol solution was prepared containing 0.322 g orcinol monohydrate (or 0.282 g orcinol anhydrous), 12 mL distilled water and 88 mL solution A. The reagent was prepared freshly on the day of testing.

Glucose was used as the standard hexose for this assay. A stock solution containing 200 µg of glucose in 0.5 mL of the protease

inhibitor solution (section 3.3.1). Dilutions of this solution were made to produce standards containing 20 - 120 µg hexose in 0.5 mL. A sample (0.5 mL) of each standard was pipetted in to a test tube followed by 2 mL orcinol solution and the contents were mixed by vortexing. The samples were incubated at 80°C for 15 min in a shaking water bath. The samples were cooled to room temperature and the absorbance was read at 540 nm against a reagent blank prepared from 0.5 mL of the protease inhibitor solution and 2 mL of orcinol solution. Each standard was determined in duplicate.

A sample of mucus (0.05 g) was weighed accurately in to a test tube. A sample (5 mL) of protease inhibitor solution as described in section 3.2.1 was then added and the samples mixed to disperse the mucus. Samples (0.5 mL) of the suspension were transferred to a separate test tube and 2 mL of the orcinol solution added. The mixture was vortexed, then incubated at 80°C for 15 min then cooled to room temperature. After this time the absorbance at 540 nm was determined with the protease inhibitor solution employed as the reference solution. Experiments were performed in triplicate for each batch of mucus tested. Hexose concentrations in the mucus samples were determined by interpolation from the linear calibration curve obtained by linear regression.

3.3.3 Rheological evaluation

Rheological evaluation of the mucus was performed at either Sturge-Lifford Chemical company on a Carri-med CS500 rheometer or at Brighton Polytechnic with a Carri-med CS100 instrument. The rheometer and computer were set up using the parameters given in table 3.1 (El-Hariri, 1989).

The mucus sample was placed on the ram below the 4 cm diameter parallel plate. The ram was then raised and the sample allowed to equilibrate for 1 min before the sinusoidally oscillating voltage was applied. The frequency of oscillation was increased from 0-10 Hz by the computer, during the course of the experiment. After the test was complete the ram was lowered, the mucus removed and the cone cleaned

Table 3.1 Experimental rheological parameters used to determine the viscosity and elasticity of mucus.

parameter	value
plate geometry	4 cm parallel
liquid density	1.00 gcm^{-3}
equilibration time	1.00 min
machine inertia	22.42 μ Nm s^{-2}
measurement system factor	1.989 x 10^{-3}
fluid density factor	8.333 x 10^{-3}
measurement system gap	500 μm
auto amplitude	6.000 milliradians
oscillatory mode	frequency sweep
temperature	20°C
torque	100.0 μ Nm
start frequency	1.000 Hz
final frequency	10.00 Hz
number of points	10
generating software	4.3 oscillation

with acetone. The procedure was repeated at least three times with new samples of mucus.

3.3.4 To determine the effect of concentrating mucus by ultrafiltration and centrifugation

The pig gastric mucus was obtained and treated with protease inhibitor solution as described in section 3.3.1. After dialysis half the mucus sample was reconcentrated to its original volume by centrifugation (as already described) and the remainder was reconcentrated by ultrafiltration.

The biochemical and rheological properties, as described above, of the two batches of mucus were then determined.

3.3.5 To determine the effect of freezing on the reconcentrated mucus.

Samples of reconcentrated mucus were frozen at -20°C. After 1 week, the sample was removed from the freezer and allowed to defrost at room temperature. Rheological evaluation of the mucus was then determined.

3.4 RESULTS AND DISCUSSION

The aim of this work was to develop and test a method of treatment and storage of pig gastric mucus, which was representative of mucus *in vivo* and which could subsequently be used to assess the importance of mucus as a barrier to peptide drug delivery.

Mucus was obtained from the stomachs of recently slaughtered pigs by scraping from the mucosal surface then mixing with an equal volume of a protease inhibitor solution in order to reduce proteolysis of the glycoprotein molecules and the associated loss of gel structure (Scawen and Allen, 1977; Bell et al., 1985). The samples were dialysed overnight against the protease inhibitor solution then reconcentrated to the original volume by either centrifugation or ultrafiltration. The mucus so prepared was characterised by biochemical analysis of dry weight, protein and hexose content and rheological evaluation.

The results of the biochemical analysis of eleven different batches of mucus are tabulated in table 3.2. They indicated that the reconcentrated mucus had a mean dry weight of 7.07 % w/w, with a standard deviation of 1.23 % and a range of 3.96%. This was determined by drying the sample, at 100°C, to constant weight. The mean value was not appreciably different to the value of 8.34 ± 0.68% obtained for crude, untreated mucus and correlated well with the water content of 90-95 % reported by other workers (Creeth, 1978)

Table 3.2 Dry weight (% w/w of mucus) determinations for different batches of mucus obtained after drying to constant weight at 100°C. Data are the mean ± SD_{n-1} of three determinations. Overall mean is the mean of thirty three determinations.

batch	dry weight % w/w of mucus
A	6.76 ± 0.69
B	9.27 ± 0.45
C	7.28 ± 1.29
D	5.87 ± 0.12
E	5.31 ± 0.17
F	7.47 ± 1.33
G	7.61 ± 0.17
H	7.51 ± 0.15
I	5.66 ± 0.13
J	6.39 ± 0.56
K	8.65 ± 0.37
overall mean	7.07 ± 1.23
native mucus	8.34 ± 0.33

The results of the protein determination of the eleven batches of mucus, determined by the method of Bradford (1976) are presented in table 3.3. The protein measured, relative to BSA, will be a total of secreted and transudated proteins, proteins comprising the glycoprotein molecule and additional proteins incorporated by scraping the mucus from the underlying mucosa and from bacteria. Proteins of varying molecular weights and amino acid composition will be present in mucus and may interact with dye molecules to different degrees. The determined protein value will be relative to BSA, and will be a relative, rather than an absolute value. The content of free protein in mucus is typically 0.5-1% by weight free protein (Creeth, 1978) and 0.5-5% by weight glycoproteins (Carlstedt et al., 1985). The mean protein content of the eleven different batches of mucus, relative to BSA, was 1.58 % w/w of mucus with a standard deviation of 0.55 and a range of 0.68 to 2.42 % w/w of mucus.

The hexose content was determined by a modified orcinol method (Winzler, 1955) with glucose as standard. The results of the hexose determination of eleven batches of mucus are presented in table 3.3. The mean hexose content was 0.69 % w/w of mucus, with a standard deviation of 0.15 and range of 0.47 to 0.96 % w/w of mucus.

Table 3.3 Protein and hexose content (% w/w of mucus) of eleven different batches of mucus and the calculated hexose/protein ratio. Data are the mean ± SD_{n-1} of three determinations.

Batch	Protein Mean conc (w/w %)	Hexose Mean conc (w/w %)	Hexose/protein ratio
A	1.14 ± 0.22	0.70 ± 0.19	0.61 ± 0.08
B	1.23 ± 0.81	0.96 ± 0.08	1.00 ± 0.42
C	1.92 ± 0.49	0.63 ± 0.10	0.34 ± 0.07
D	1.75 ± 0.22	0.59 ± 0.08	0.35 ± 0.08
E	2.02 ± 0.24	0.77 ± 0.44	0.38 ± 0.16
F	2.41 ± 0.14	0.92 ± 0.30	0.38 ± 0.09
G	1.90 ± 0.33	0.65 ± 0.22	0.36 ± 0.18
H	2.11 ± 0.25	0.84 ± 0.03	0.40 ± 0.05
I	0.91 ± 0.13	0.47 ± 0.16	0.52 ± 0.17
J	0.68 ± 0.19	0.54 ± 0.10	0.82 ± 0.15
K	1.30 ± 0.43	0.60 ± 0.26	0.54 ± 0.39
mean	1.58 ± 0.55	0.69 ± 0.15	0.52 ± 0.22

The calculated hexose:protein ratio is often used by workers to reflect glycoprotein structure (Walters, 1986) and as a means of comparing mucus secretions (Brofeldt et al., 1986). The basis of this analysis is the knowledge that glycoprotein molecules comprise 70-85% carbohydrate by dry weight (Gallagher and Corfield, 1978; Allen, 1983). Therefore, for a pure glycoprotein molecule the hexose:protein ratio would be 3-5 and lower values would suggest a high degree of non-glycoprotein protein, possibly due to secretory proteins such as lactoferrin, plasma contamination (Readman, 1983) and from cellular material introduced during the collection period (Bell et al., 1985).

Although it is acknowledged that total hexose and protein levels of crude mucus do not truly reflect glycoprotein structure, the hexose:protein value is a useful indication of the glycoprotein contribution to the secretion (Readman, 1983). The hexose:protein ratios determined for the different batches of mucus are illustrated in table 3.3.

The mean hexose:protein ratio of all eleven batches was determined to be 0.52 with a standard deviation of 0.22 and a range from 0.34 to 1.00. Walters (1986) reported a hexose:protein value of 2.82 for a purified high molecular weight glycoprotein fraction and 0.2 for the fraction comprised of lower molecular weight protein and smaller glycoprotein molecules.

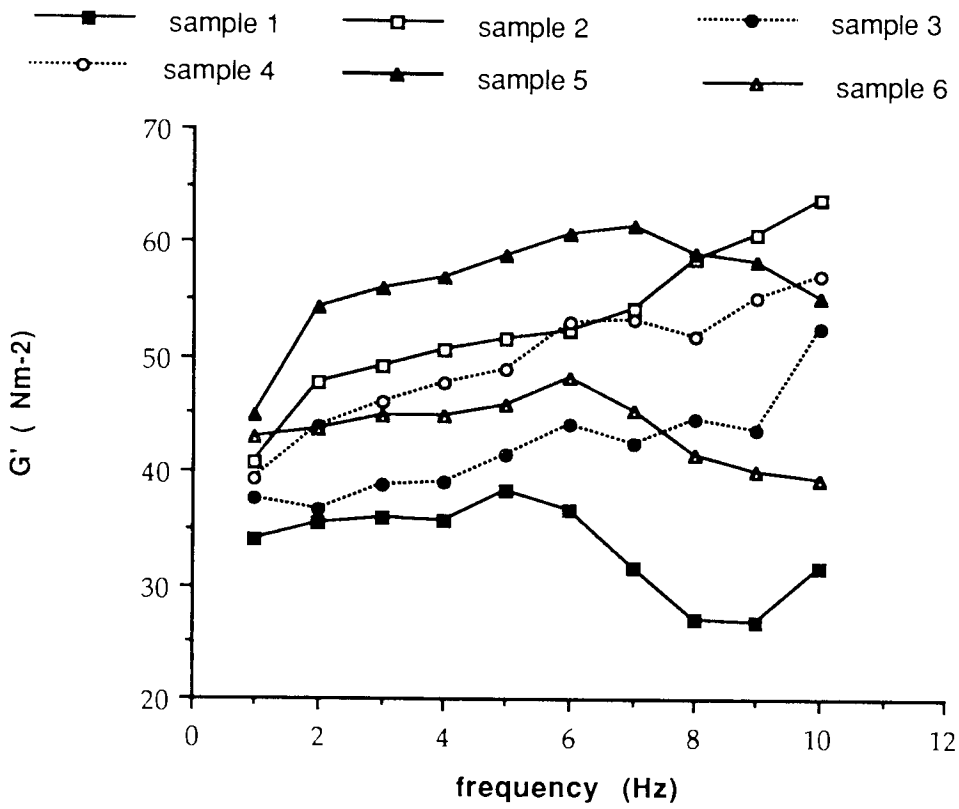
The mucus in this preparation will contain high molecular weight undegraded glycoprotein, some lower molecular weight glycoproteins from the lumen surface, where proteolytic degradation will have been greatest and additional proteins. These components would explain the intermediate value of 0.52 being obtained. The ratio values are similar to those obtained for nasal secretions (Brofeldtet al., 1986).

Rheological evaluation was performed using a Carri-med rheometer in oscillatory mode to determine the viscous and elastic properties of the mucus preparation. The rheological parameters determined were the G', the storage (elastic) modulus and G'', the loss (viscous) modulus using the procedures outlined in section 3.2

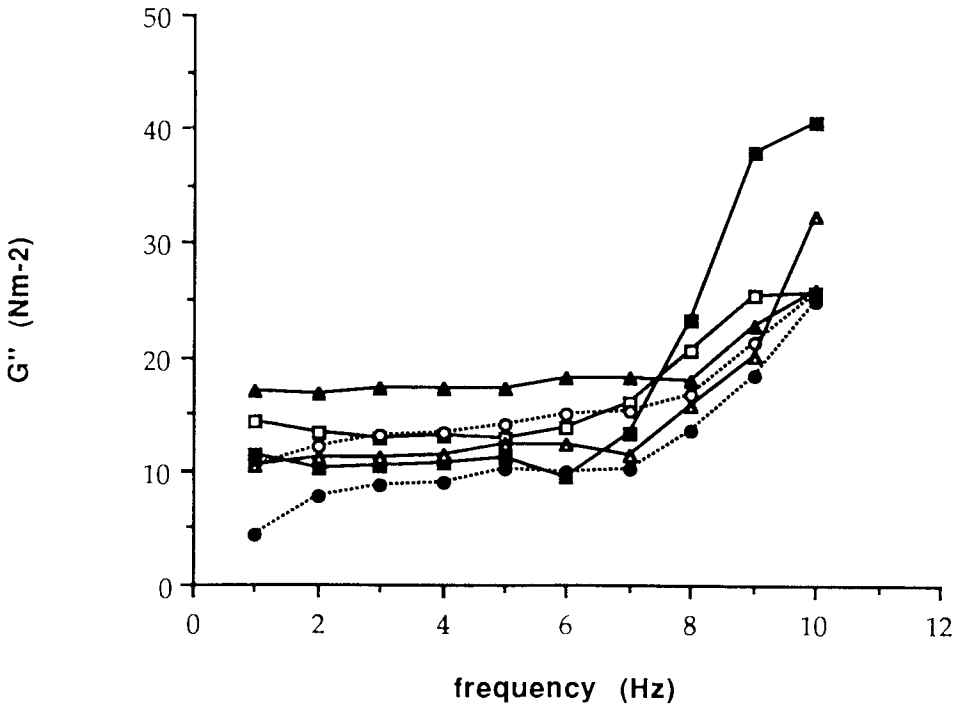
Figures 3.4 a and b indicate the large variation obtained in G' and G'' respectively, for six different samples of the same batch of mucus. This variation is consistent with observations of other workers. Large inter- and intra-subject variation have previously been reported in determined rheological parameters for fresh human cervical mucus (Litt et al., 1976), tracheal mucus from the mini-pig (Martin et al., 1990) and in pig gastric mucus (El-Hariri, 1989).

Figure 3.4 To illustrate the variation in a) G' and b) G'' for six samples of the same batch of mucus. Rheological evaluation was performed with the conditions outlined in table 3.1.a)

a)



b)



The mean values of the storage modulus, G', were considerably higher than those obtained for the loss modulus,G'', throughout the frequency range employed (0-10 Hz) in agreement with previous results for pig gastric mucus (Bell et al., 1985; Walters, 1986). This behaviour is characteristic of a gel network (Bell et al., 1985). G' was observed to increase with increasing frequency, from 39.94 ± 3.93 Nm^{-2} at 1 Hz to 50.01 ± 12.07 Nm^{-2} at 10 Hz, but the mean values at different frequencies were not appreciably different to each other. The loss modulus similarly increased from 11.35 ± 4.33 Nm^{-2} at 1 Hz to 17.98 ± 3.52 Nm^{-2} at 8 Hz. At a frequency of 8 Hz the G'' values began to rise rapidly to a value of 29.27 ± 6.24 Nm^{-2} at 10 Hz.

The mean value of G', at 5 Hz was 47.46 Nm^{-2}, with a standard deviation of 7.40. The mean value of G'' was 13.01 Nm^{-2} at 5 Hz and had a standard deviation of 3.25. The values of G' were within the range of 20-48 Nm^{-2} reported by Bell et al. (1985) for native pig gastric mucus, obtained by scraping the stomachs of freshly slaughtered pigs, but were smaller than the values obtained by El-Hariri (1989), which are presented in table 3.4. These results may be higher due to the method of preparation and the considerably higher (12% w/w) dry weight of the samples. The G' value obtained for the purified gel was however markedly lower. The G'' values obtained in this study were higher than those reported by Bell et al. (1985) but not significantly different to those reported to those of El-Hariri (1989).

Table 3.4 Rheological parameters determined for a purified glycoprotein gel (pH 7.4) (PGG) and 12 % w/w native mucus at pH 7.0 (NM) at 5 Hz. The native mucus was prepared in the following manner. The stomachs of recently slaughtered pigs were obtained, washed and frozen. Prior to use the stomachs were defrosted, the mucus scraped form the mucosa, pooled , pH adjusted and ultrafiltrated. The samples were adjusted to give a 12 % w/w dry weight (El-Hariri, 1989).

Sample	G' Nm^{-2}	G'' Nm^{-2}	Number of tests
PGG	28.9 ± 2.70	17.4 ± 2.4	6
NP	71.4 ± 10.8	17.7 ± 2.1	12

One batch of mucus, which had been treated with the protease inhibitor solution and subsequently dialysed overnight, was divided into two portions. One was reconcentrated by ultrafiltration and the other by centrifugation. The results are illustrated in figure 3.5. The different methods employed to reconcentrate the mucus samples did not exert a marked effect on the rheological parameters. The values of G' and G'' for the two samples are tabulated in table 3.5.

Figure 3.5 To illustrate the effect of reconcentrating mucus by ultrafiltration and centrifugation on the rheological parameters of the secretion. Ultrafiltration was performed with an Amicon 8200, stirred ultrafiltration cell, fitted with Diaflo PM10 membranes. Centrifugation was performed at 20,000 *g* for two x 30 min periods at 4°C. Points are the mean ± sem of six determinations.

a)

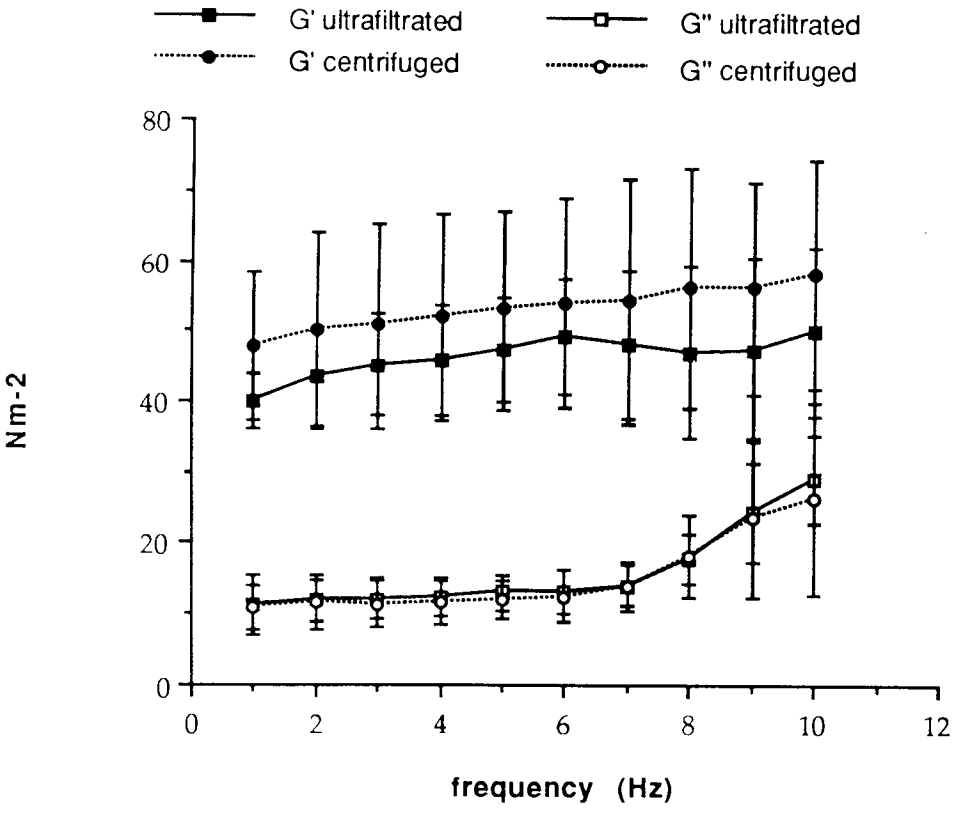


Table 3.5 Rheological parameters determined for the same batch of mucus reconcentrated by either ultrafiltration (UF) or centrifugation (CEN) at 5 Hz. Ultrafiltration was performed with an Amicon 8200, stirred ultrafiltration cell, fitted with Diaflo PM10 membranes. Centrifugation was performed at 20,000 *g* for two x 30 min periods at 4°C.

Sample	G' Nm^{-2}	G'' Nm^{-2}	Number of tests
UF	47.47 ± 7.40	13.01 ± 2.50	6
CEN	53.08 ± 14.12	12.01 ± 2.56	6

It was decided therefore to reconcentrate all samples by centrifugation, as this method was more rapid than ultrafiltration and, additionally, meant that food debris could easily be removed from the preparation, in the form of a pellet at the bottom of the centrifuge tubes.

Freezing the mucus and then defrosting the sample at room temperature caused the mucus to separate into two distinct phases, one watery and of lower viscosity and the other of higher viscosity. It was found impossible to re-mix the two layers to reform the original gel structure by stirring. Rheological evaluation of the more solid component of the thawed mucus, showed it to have considerably higher values of both G' (100.32 ± 8.35 Nm^{-2} at 5 Hz) and G'' (23.61 ± 15.00 Nm^{-2} at 5 Hz). This may be due to the higher dry weight of the sample due to exclusion of some of the water. The dry weight was however not determined. A similar result was obtained if the mucus had been reconcentrated by either ultrafiltration or centrifugation.

The rheological parameters of three batches of mucus were kindly determined at Brighton Polytechnic by L. El-Hariri after preparation at Aston and delivery in the post. The samples were typical of those

prepared in this laboratory, with the gels exhibiting viscosity and elasticity, but they were reported to be visibly less viscous than crude pig gastric mucus upon arrival. The values of G' and G'' for all three batches of mucus were appreciably lower than reported values (Bell et al., 1985; El-Hariri, 1989).

The mean values for G' at 5 Hz being 24.10 ± 7.8 Nm^{-2}, 14.82 ± 2.01 Nm^{-2} and 27.79 ± 7.58 Nm^{-2} . The values for G'' were calculated to be 7.93 ± 1.80 Nm^{-2}, 7.86 ± 0.48 Nm^{-2} and 3.96 ± 1.42 Nm^{-2}. The visible observations and rheological measurements suggested that the samples were not typical of pig gastric mucus. It was proposed that the nature of the mucus had been damaged in the unrefrigerated transit to Brighton, possibly due to microbial degradation. A similar, visible loss of viscosity was observed if samples were stored at 4°C for periods longer than 8 days. Samples which were subsequently analysed at Brighton were refrigerated in transit and the samples were reported to "look right" and have G' and G'' typical of pig gastric mucus (data not shown).

In view of these observations it was concluded that in order for the mucus to possess its characteristic viscoelastic nature the reconcentrated samples could not be frozen and had to be used within eight days of collection.

In conclusion, the mucus preparation was considered to be a suitable model of the in vivo secretion on the basis of biochemical and rheological analysis. The method of centrifugation was chosen to reconcentrate the mucus after dialysis in preference to ultrafiltration. Mucus samples were prepared freshly each week of use and the dry weight, protein and hexose content determined as an indication of the composition of the secretion in relation to other batches.

3.4 SUMMARY

A model of mucus, based on native pig gastric mucus, treated with protease inhibitors to reduce degradation, was evaluated by biochemical and rheological means to assess its suitability as a model of *in vivo* mucus. This was carried out in order to assess the importance of this biological barrier to peptide drug delivery.

The biochemical evaluation indicated that the eleven batches of pig gastric mucus had a mean dry weight of 7.07 ± 1.23 % w/w, a protein content of 1.58 ± 0.55 % w/w a hexose content of 0.69 ± 0.15 % w/w. The hexose:protein ratio had a mean value of 0.52 ± 0.22. These values were in agreement with values reported by other workers for native pig gastric mucus.

The rheological evaluation indicated that the storage (elastic) modulus G' values (47.47 ± 7.40 Nm^{-2} at 5 Hz) were higher than those for the loss (viscous) modulus, G'' (13.01 ± 2.50 Nm^{-2}), throughout the frequency range and were characteristic of gels and of experimental values reported for pig gastric mucus by other workers.

The two methods of reconcentrating the mucus to its original volume, ultrafiltration and centrifugation were evaluated and were found to produce no appreciable difference in the rheological nature of the secretions. Centrifugation was therefore chosen to prepare subsequent batches. Freezing the mucus and storage for longer than eight days at 4°C, were, however, shown to cause marked changes in these properties.

In conclusion, the mucus prepared freshly by this method and used within eight days of collection, was taken to be a suitable model. The biochemical content of subsequent batches was determined as an indication of mucus content.

CHAPTER 4
DIFFUSION THROUGH MUCUS

CHAPTER 4

DIFFUSION THROUGH MUCUS

4.1 INTRODUCTION

One of the earliest investigations of diffusion through mucus was performed by Heatley (1959). It was shown that there was no significant difference between diffusion of hydrochloric acid and pepsin through pyloric and duodenal mucus in comparison to diffusion through an unstirred aqueous layer of the same thickness. Therefore it was assumed that it was the stationary nature of this layer that was of the greatest importance to drug delivery rather than any interactions between drug and molecules comprising the mucus gel. Diluted mucus preparations were however used in this study at non-physiological concentrations.

This idea remained undisputed until the role of gastric mucus in cytoprotection was investigated. Several studies have subsequently been performed to investigate the diffusion of hydrogen ions through mucus from different species located at a variety of anatomical sites. All of the studies demonstrated that the diffusion of hydrogen ions was significantly slower through a mucus layer than through an aqueous unstirred layer of the same thickness.

Pfeiffer (1981) observed a two-fold reduction in the rate of diffusion of hydrogen ions using three-compartment diffusion cells and mucus collected from the rabbit small intestine, pig stomach, rat intestine and human colon. A similar, 2-fold reduction was also observed by Winne and Verheyen (1990) who employed rat small intestinal mucus and both three-compartment diffusion cells and capillary methodology. A larger, 5-fold reduction was reported by Turner et al. (1985) employing native porcine gastric mucus and three-compartment diffusion cells, whilst a 10-fold reduction has been reported by Slomiany et al. (1986) with pig gastric mucus and Sarosiek et al. (1984) with dog gastric mucus.

It has also been shown that mucus can significantly retard the diffusion of other small, charged ions. In the case of potassium, the equilibrium concentration was attained in the diffusion cell within twenty minutes in the absence of mucus. The imposition of a mucus gel, however, reduced diffusion to a slow linear rate with the final concentration after 2 hours being only 8 % of the equilibrium value (Lee and Nicholls, 1987). For sodium, the diffusion was observed to be dependent on the mucin concentration. A 2-fold reduction in the rate of diffusion was observed at a 10% w/v concentration of artificial porcine mucus increasing to a 9-fold reduction at 30% w/v concentration (Lucas, 1984).

Experiments performed with mucus from other species have also demonstrated that mucus is a greater barrier to diffusion than an unstirred aqueous layer. These range from small organic molecules such as butyrate through pig colonic mucus (Smith et al., 1986) to the larger, more complex ergot alkaloids diffusing through rat small intestinal mucus (Nimmerfall and Rosenthaler, 1980).

Several different classes of antibiotics have been investigated in relation to respiratory infection most notably with regard to cystic fibrosis. These include a survey of eighteen different antibiotics through artificial porcine gastric mucin (Saggers and Lawson, 1966), tetracycline through porcine gastric mucus (Braybrooks at al., 1975; Kearney and Marriott, 1987) and benzylpenicillin potassium and clavulanic acid through both native porcine mucus and a purified mucus glycoprotein gel (Cheema, 1985). The diffusion of two β-lactams ticarcillin and piperacillin through purified sputa obtained from cystic fibrosis patients has also been evaluated (Bolister, 1989).

A few studies have investigated the diffusion of a series of chemically different compounds to elucidate whether particular chemical features exert an effect on the rate of diffusion with a variety of conclusions obtained (Hughes, 1988; Winne and Verheyen, 1990; Desai and Vadgama, 1991).

The role of mucus as a barrier to peptide delivery has been postulated but not explored experimentally. The mucus barrier may be of particular importance to peptide and protein molecules due to mucus function and to peptide:glycoprotein interactions. It has been suggested that one of the prime roles of mucus is to exclude macromolecules from contact with cell membranes (Edwards, 1978) which is believed to be a function of its viscosity and electronegative charge (Humphrey, 1986a).

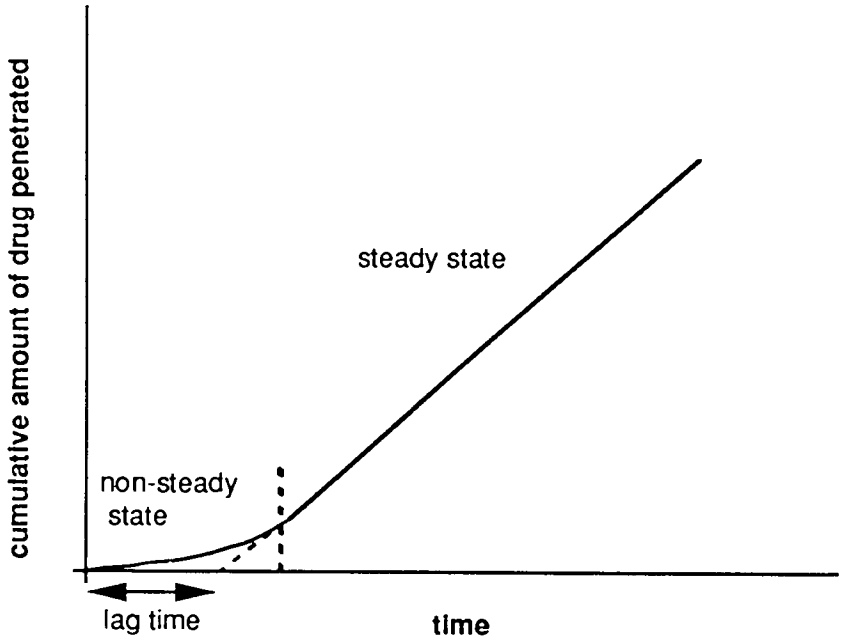
Some of the peptide:glycoprotein interactions already known include increasing the viscosity of mucus (Marriott, 1981) and eliciting its secretion (Gallagher and Richardson, 1981). These are properties which will increase the barrier to absorption by increasing the thickness of the mucus layer and making diffusion through it more difficult. Analogous to this is the action of bile salts and mucolytics such as N-acetylcysteine upon mucus which reportedly reduce the viscosity of mucus (Martin et al., 1978; Livingstone et al, 1990). The effects of mucolytics on diffusion through mucus will be discussed in chapter 5.

In view of the fact that peptides are generally high molecular weight species, are charged and are hydrophilic it was decided to assess whether the barrier properties of mucus were mechanical, chemical or a combination of both. The effect of increasing molecular weight, charge, lipophilicity and chemical group was investigated with the compounds tryptophan (TRP), tryptophylglycine (WG), tryptophylglycylglycine (WGG); benzoic acid (BA), β-blockers (propranolol (PR), oxprenolol (OX) and atenolol (AT)) and N-acetylphenylalanine (NP) respectively.

4.2 THEORETICAL TREATMENT OF RESULTS

A typical diffusion profile of a drug diffusing through a mucus layer sandwiched between two synthetic membranes is illustrated in figure 4.1. It is characterised by a period of non-steady state diffusion (non-linear portion of graph) followed by steady-state penetration (linear portion of graph) where there is a net balance in the rate of entry and exit of drug into and out of the mucus layer.

Figure 4.1 Schematic representation of a typical diffusion profile through mucus using a three-compartment side-on diffusion cell.



The most simple way of modelling the diffusion process is the application of Fick's first law of diffusion to the steady-state phase. The law states that the rate of transfer of a diffusing substance through a unit area of a section (the flux) is proportional to the concentration gradient across the entire barrier phase.

$$J = - D \frac{dc}{dx} \qquad (4.1)$$

Where J is the rate of transfer per unit area of surface (the flux), with units of μmoles cm^{-2} min^{-1}, dc/dx is the concentration gradient across the membrane and D is the diffusion coefficient with units cm^2 min^{-1}. The flux of the penetrant corresponds to the slope of the steady-state diffusion curve in figure 4.1.

For a drug diffusing across a membrane of thickness, h, the concentration gradient may be written as:

$$\frac{C_d - C_r}{-h} = - \frac{\Delta C}{h} \qquad (4.2)$$

where C_d is the concentration at the donor side of the membrane and C_r is the concentration entering the receiver compartment. By substituting for dc/dx into equation 4.1 it can be shown that:

$$J = \frac{D.\Delta C}{h} \qquad (4.3)$$

If however the barrier is not simply an inert structural barrier to diffusion but one with an affinity for the applied solute, partitioning into the membrane is also important. For this reason the parameter, P, the membrane-vehicle partition coefficient is introduced where:

$$P = \frac{C_1}{C_d} = \frac{C_2}{C_r} \qquad (4.4)$$

and C_1 and C_2 are the concentrations within the membrane, at the donor and receiver compartment side of the membrane respectively. From equation 4.4 it follows that as:

$$dc = C_1 - C_2 = PC_d - PC_r = P(C_d - C_r) = P\Delta C \qquad (4.5)$$

the flux may be rewritten as:

$$J = \frac{D.P.\Delta C}{h} \qquad (4.6)$$

It is possible to combine P, D and h into a single proportionality constant K_p, which is the permeability coefficient.

$$K_p = \frac{P.D}{h} \qquad (4.7)$$

The units of K_p are cm min^{-1}. Therefore by substituting K_p into equation 4.6 it can be shown that:

$$J = K_p.\Delta C \qquad (4.8)$$

If sink conditions apply, i.e. the depletion of the donor compartment is negligible, ΔC approximates to C_v the applied drug concentration and the concentration in the receiver phase remains effectively zero. Hence equation 4.4 becomes:

$$J = K_p.C_v \qquad (4.9)$$

Using this equation it is possible to determine the permeability coefficient, K_p, by dividing the steady-state slope of figure 4.1 by the initial concentration of drug applied to the donor compartment. For two, successive barriers in series, the permeability of each may contribute a resistance to the diffusion of drug molecules according to:

$$\frac{1}{K_{pobs}} = \frac{1}{K_{pa}} + \frac{1}{K_{pb}} \qquad (4.10)$$

where K_{pobs} is the overall, combined permeability of the two membranes, and K_{pa} and K_{pb} are the permeability coefficients of membrane a and b respectively.

Likewise, the observed permeability through an aqueous unstirred layer contained within two membranes, $K_{p\ control}$, is a composite function of the permeability of the aqueous unstirred layer, K_{pw}, and the permeability of each membrane, K_{pmb}, according to:

$$\frac{1}{K_{p\ control}} = \frac{1}{K_{pw}} + \frac{2}{K_{pmb}} \qquad (4.11)$$

A similar equation may be written for the permeability of a mucus layer contained within two membranes :

$$\frac{1}{K_{p\ mucus}} = \frac{1}{K_{pmu}} + \frac{2}{K_{pmb}} \qquad (4.12)$$

where $K_{p\ mucus}$, is the observed permeability through the mucus layer and K_{pmu} is the permeability of the mucus layer.

Prior to establishment of steady-state diffusion there is a period where the flux of the drug is gradually increasing (non-steady state) as shown in figure 4.1. The linear portion of the line can be extrapolated to the x-axis in order to define a lag time, T_L, which is dependent upon the membrane diffusion coefficient D, and the thickness of the membrane δ as shown in equation 4.13.

$$T_L = \frac{\delta^2}{6D} \qquad (4.13)$$

The determination of the lag time permits the estimation of the diffusion coefficient, providing there is no binding. It should also be noted that the equations 4.2-4.5 are only applicable to the steady-state phase provided there are no significant interactions, such as binding, between the drug and the molecules comprising the mucus gel.

4.2.1 Calculation of retardation of diffusion of mucus as a ratio

To permit the comparison of the diffusion rates through mucus of the different compounds evaluated it was necessary to produce a parameter which included the differences in flux through both the mucus and the aqueous layer. To achieve this the data for each compound was transformed in the following way. A ratio of the calculated flux through the mucus layer against the calculated flux through the aqueous control was determined by a crossover-analysis. This involved taking the flux values through mucus, determined in triplicate (A_m, B_m and C_m in table 4.1) and dividing each value by the flux values determined in triplicate for the aqueous control (A_w, B_w and C_w in table 4.1). For each compound, the mean and standard deviation of the nine ratios was then determined.

Table 4.1 A table to illustrate the crossover-analysis performed to calculate the mean and standard deviation of the mucus retardation ratios.

		aqueous control flux values		
		A_w	B_w	C_w
mucus flux values	A_m	A_m/A_w	A_m/B_w	A_m/C_w
	B_m	B_m/A_w	B_m/B_w	B_m/C_w
	C_m	C_m/A_w	C_m/B_w	C_m/C_w

The theoretical basis of this parameter is as follows. The observed permeability of the unstirred aqueous and mucus layers may be represented by equations 4.11 and 4.12. These may be rearranged to give equations 4.14 and 4.15 respectively

$$\frac{1}{K_{p\ control}} = \frac{2K_{pw} + K_{pmb}}{K_{pmb}.K_{pw}} \qquad (4.14)$$

$$\frac{1}{K_{p\ mucus}} = \frac{2K_{pmu} + K_{pmb}}{K_{pmb}.K_{pmu}} \qquad (4.15)$$

Therefore, $K_{p\ mucus}$ divided by $K_{p\ control}$ can be represented by:

$$\frac{(2K_{pw} + K_{pmb}).K_{pmb}.K_{pmu}}{(2K_{pmu} + K_{pmb}).K_{pmb}.K_{pw}} \qquad (4.16)$$

If the membrane is much more permeable than the aqueous or mucus layer, (i.e. $K_{pmb} \gg K_{pw}$ and $K_{pmb} \gg K_{pmu}$), equation 4.16 may be reduced to:

$$\frac{K_{p\ mucus}}{K_{p\ control}} = \frac{K_{pmb}.K_{pmb}.K_{pmu}}{K_{pmb}.K_{pmb}.K_{pw}} \qquad (4.17)$$

which can be written as:

$$\frac{K_{p\ mucus}}{K_{p\ control}} = \frac{K_{pmu}}{K_{pw}} \qquad (4.18)$$

which is the ratio of permeability of mucus to the permeability of the aqueous unstirred layer.

The assumption that $K_{pmb} \gg K_{pw}$ and $K_{pmb} \gg K_{pmu}$, leading to equation 4.18, was found to be correct for the diffusion of TRP. Ratios were therefore determined for all other compounds using the cross-over analysis described above.

4.2.2 Statistical analysis

Statistical analysis was performed on the mucus retardation ratios using the unpaired-sample t-test, described by equation 4.19 (Bolton, 1984).

$$t = \frac{\bar{X}_1 - \bar{X}_2}{(\sigma_1^2/N_1 + \sigma_2^2/N_2)^{1/2}} \qquad (4.19)$$

Samples were tested at the 5% significance level using 2-tailed analysis.

4.3 EXPERIMENTAL

4.3.1 Design of the Diffusion Cell

Three different designs of side-on three-compartment diffusion cells were evaluated, and upgraded to produce the final diffusion cell used for the diffusion experiments. The first cell (A), was constructed of glass and comprised two compartments which each held 30 mL of solution. The mucus layer was sandwiched between two Nuclepore polycarbonate membranes (0.45 μm pore size) at a thickness of 5 mm as defined by the polypropylene spacer disc. The diffusional area was 3.14 cm^2. The three compartments were held together by two steel springs which ran lengthwise along the cell. The cell had an external glass jacket for maintenance of constant temperature.

Several problems were encountered with the cell. Firstly, the large volume required to fill the compartments had financial implications in view of the cost of the compounds to be investigated in diffusion experiments. The thickness of the mucus layer was also large and it was decided to try and reduce this. The major problem however was the difficulty in assembling the apparatus so that it did not leak during the course of the experiment. It was therefore decided to introduce more glass hooks so that the cell could be held together by a greater number of steel springs. Another manipulative problem was the difficulty in positioning the cells on the bench due to the cell being completely round. It was decided to flatten the lower surface of the external jacket to enable it to balance securely on the bench top.

Cell B was constructed introducing some of the modifications described above. The cell volume was reduced to 26 mL, which although smaller was still considered to be too large. It was noted however that

during experiments performed with this cell that the magnetic fleas regularly ceased to stir the media but were both attached to the membrane. The cessation of stirring significantly reduced the rate of transport and it was decided to introduce a small well into each compartment to contain the magnetic fleas and circumvent this problem. The increased number of springs reduced but did not completely eliminate the leakage problem.

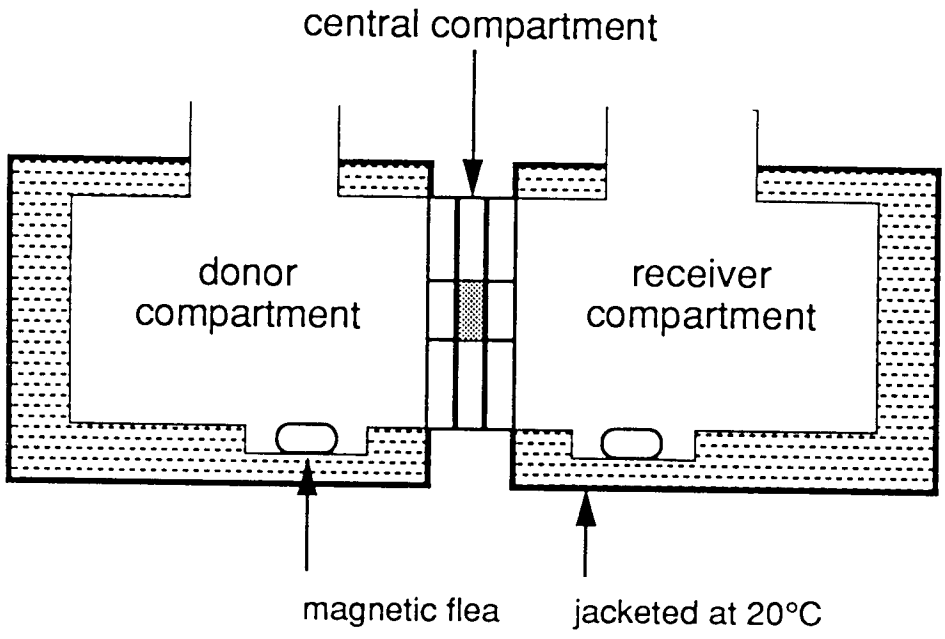
The third cell prototype had a compartmental volume of 10 mL, which was considered suitably small for experiments. The purchase of a smaller thickness polypropylene sheet enabled the mucus layer to be reduced to 1 mm. The introduction of the wells to contain the magnetic fleas eliminated the problem of the stirrers becoming attached to the membranes.

Experiments performed with this cell demonstrated that it was difficult to obtain repeatable results with either buffer in the central compartment or mucus. It was noted that during the course of the experiment the membranes appeared to bulge outwards. which would alter the length of the pathway in the central compartment and additionally alter the surface area for transport to occur across. These problems were overcome by introducing two additional polypropylene discs (membrane support discs) each with an inset gauze filter which rested against the membrane, either side of the spacer disc. These extra discs overcame both the membrane and the leakage problem. The final cell design is illustrated in figure 4.2.

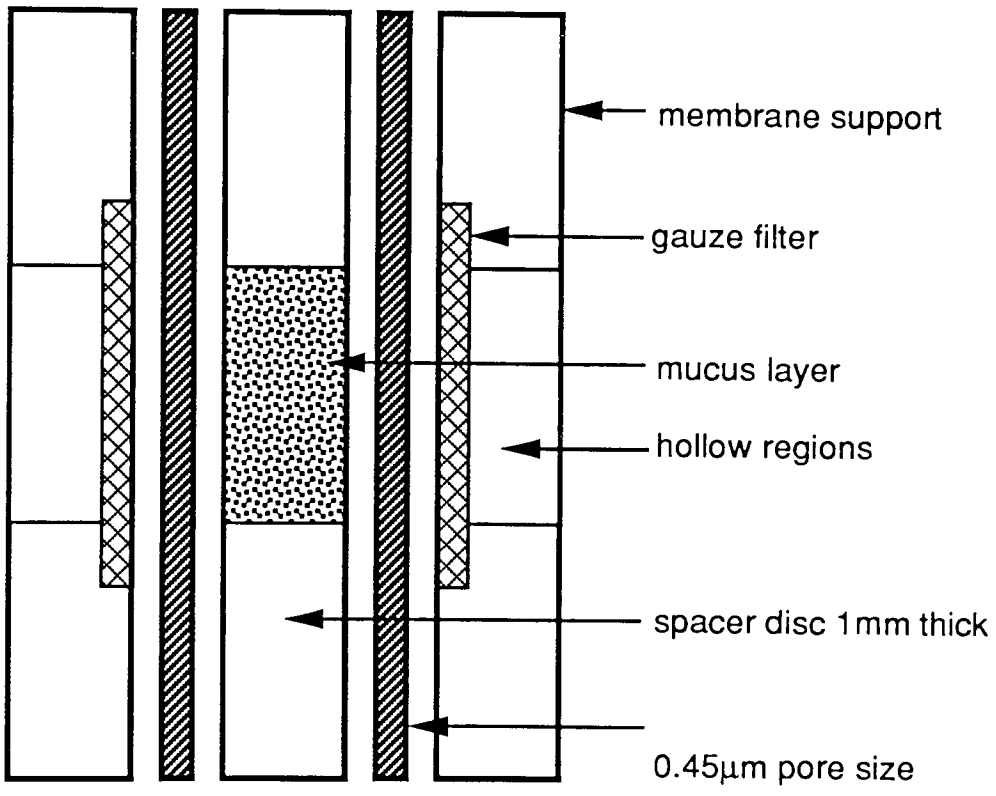
4.3.2 Assembly of cell

Vaseline was sparingly applied to the roughened edges of the donor and receiver compartments and to each side of the polypropylene membrane supports and the spacer disc. The receiver compartment was placed end up on the bench and membrane support disc (containing an open-mesh gauze filter) placed on top. A Nuclepore polycarbonate membrane with 0.45 μm pore size was positioned above this and the procedure was repeated for the donor compartment. The 1 mm spacer disc was then positioned above the Nuclepore membrane resting on the receiver

Figure 4.2 Diagram of side-on three-compartment diffusion cell.



central compartment



compartment and sufficient mucus added to fill the volume. In the case of the aqueous control the addition of mucus was omitted, to allow buffer solution to diffuse into this compartment after cell assembly. A second membrane and support disc was placed above the mucus layer to complete the central compartment. The donor compartment was then placed on the central compartment. The outside of the cell was carefully wiped to remove any displaced mucus. The cell was initially sealed by winding parafilm tightly around the circumference of the cell, then clamped together with four steel springs running across the length of the cell.

The whole, intact diffusion cell was clamped horizontally above a Camlab Variomag multipoint HP magnetic stirrer with the donor compartment positioned above one stirring position and the receiver compartment above another. The use of this stirrer which permitted the cells to be stirred at the same rate in each experiment was a very large factor in achieving repeatable results. Each cell was connected serially to a Churchill circulating water pump to maintain a constant temperature of 20°C. This temperature was chosen to reduce degradation of the mucus gel which would occur at physiological temperature.

4.3.3 Experimental protocol

4.3.3.1 Mucus experiments

Phosphate buffer (pH 7.4) was added to the receiver compartment as two 5 mL and one 0.5 mL aliquots with Gilson automatic pipettes to give a total volume of 10.5 mL. The cells were carefully tilted to remove any air bubbles formed at the membrane surface. An equal volume (10.5 mL) of the drug containing phosphate buffer was then added to the donor compartment and any air bubbles formed were again carefully removed. A magnetic flea was added to each half of the diffusion cell and the Variomag multipoint magnetic stirrer switched on at a stir rate of 100 rpm. The sampling ports of both the receiver and donor compartments were sealed with parafilm to prevent evaporation.

At set time points (usually every 40 min up to 480 min) 0.5 mL samples were removed from the receiver compartment and this was replaced by an equal volume of phosphate buffer. This procedure produced a dilution of the receiver phase. It was necessary, therefore, to adjust each successive sample concentration for the dilution produced by all previous samples. This was performed by applying the following correction factor:

$$C_t = C_{mt} + V_s \frac{\sum_{t=1}^{t=n-1} C_m}{V_r} \qquad (4.7)$$

where C_t is the true concentration of drug in the receiver phase at time t, which would be found without dilution, C_{mt} is the current *measured* concentration of drug in the receiver compartment, V_s is the volume of sample removed for analysis, V_r is the volume of the receiver medium, and ΣC_m is the summed total of the previous measured concentrations (t = 1 to n-1).

This correction factor was applied to experimental data using a basic computer program written by E.J.B Holbrook, which also converted the molar concentrations to μ moles cm^{-2} membrane.

4.3.3.2 Aqueous control

In the case of the aqueous control a 0.31 mL sample of phosphate buffer (pH 7.4) was added to the receiver compartment, which diffused into and filled the central compartment and created the aqueous unstirred layer. The procedure thereafter was the same as described for the mucus experiments. These experiments will be referred to hereafter as control.

4.3.4 Repeatability and reproducibility

To determine the repeatability of diffusion through mucus the diffusion of 0.025M tryptophan (TRP) through three different cells using the same batch of mucus was evaluated on two consecutive days.

In order to assess the reproducibility of diffusion through mucus the diffusion of 0.025M TRP was determined through three different batches of mucus obtained and purified at three different times.

Samples (100 µL) of each time sample were transferred to a separate test tube to which 100 µL of I.S. solution (phenylalanine 5 mM) were added and the mixture made upto 1 mL with phosphate buffer (pH 7.4). Analysis was performed with HPLC as described in section 2.3.1.1

4.3.5 To determine the ability of mucus to retard the diffusion of tryptophan

To determine the contribution of the Nuclepore polycarbonate membrane on diffusion the following experiment was performed. A single Nuclepore membrane was positioned between the donor and receiver compartment. Phosphate buffer (10.5 mL) was then added to the receiver compartment and air bubbles carefully removed from the membrane by tilting the cell. 10.5 mL phosphate buffer containing 0.025M TRP was then added to the donor compartment. The procedure thereafter was the same as described in section 4.3.3.1 except the samples were taken every 15 min up to 180 min. The experiment was performed in triplicate.

The mucus and control experiments for 0.025M TRP were carried out as described in sections 4.3.3.1 and 4.3.3.2 respectively. The experiments were performed at least in triplicate. Samples (100 µL) of each time sample were transferred to a separate test tube to which 100 µL of I.S. solution were added and the mixture made up to 1 mL with phosphate buffer (pH 7.4). Analysis was performed with HPLC as described in section 2.3.1.1

4.3.6 Diffusion of a variety of compounds through mucus

It was decided to investigate the effect of increasing molecular weight, charge, lipophilicity and chemical group on the rate of diffusion through mucus. To assess these parameters a range of compounds was selected.

The effect of molecular weight was investigated with an aromatic amino acid tryptophan (TRP), a dipeptide tryptophylglycine (WG) and a tripeptide tryptophylglycylglycine (WGG). The effect of charge was investigated by comparing the diffusion of the negatively charged carboxylic acid benzoic acid (BA) in comparison with the diffusion of three positively charged β-blocker drugs. The three β-blockers chosen were propranolol (PR), oxprenolol (OX) and atenolol (AT). These three compounds were also used to investigate the effect of lipophilicity on the rate of diffusion through mucus with PR being very lipophilic (Log P = 3.21), OX lipophilic (Log P = 2.37) and AT (Log P = 0.16).

The effect of the carboxyl and amino chemical group on the rate of diffusion was investigated with compounds which had either a blocked amino or carboxyl group these were N-acetylphenylalanine (NP) the methyl ester of phenylalanine (ME) respectively.

In each case the donor solution was 0.025M and the experimental procedures were as described in sections 4.3.3.1 and 4.3.3.2. Experiments were performed at least in triplicate. Samples (100 µL) of each time sample were transferred to a separate test tube to which 100 µL of I.S. solution was added and the mixture made upto 1 mL with phosphate buffer (pH 7.4). Analysis was performed with HPLC as described in section 2.3.1.

4.3.6.1 The hydrolysis of the methyl ester of phenylalanine

A 0.025M solution of the methyl ester of phenylalanine (ME) in phosphate buffer (pH 7.4) was prepared in a volumetric flask. The solution was transferred to a stoppered glass flask, which was immersed in a water bath at 20°C. The flask contents were stirred with a magnetic flea. Samples (0.5 mL) were removed every 10 min for the first hour then every 30 min up to 10 h.

The concentration of ME was determined in the samples taken up to 6 h using the HPLC methodology developed for this compound, which is described in section 2.3.1.9. Using this mobile phase it was impossible to determine the concentration of PHE as this compound was

unresolved from the solvent front. The concentration of phenylalanine was determined in the samples taken after 330 min using the HPLC method described in section 2.3.1 Employing this mobile phase the compound ME was observed as a very broad squat peak, making quantification difficult. For this reason the ME concentrations were not determined.

4.4 RESULTS AND DISCUSSION

4.4.1 Repeatability and reproducibility of diffusion through mucus

In order to assess the variation in the rates of diffusion through mucus, the diffusion of 0.025M TRP through the same batch of mucus was evaluated in three cells on two consecutive days. The results obtained are illustrated in figures 4.3a and b.

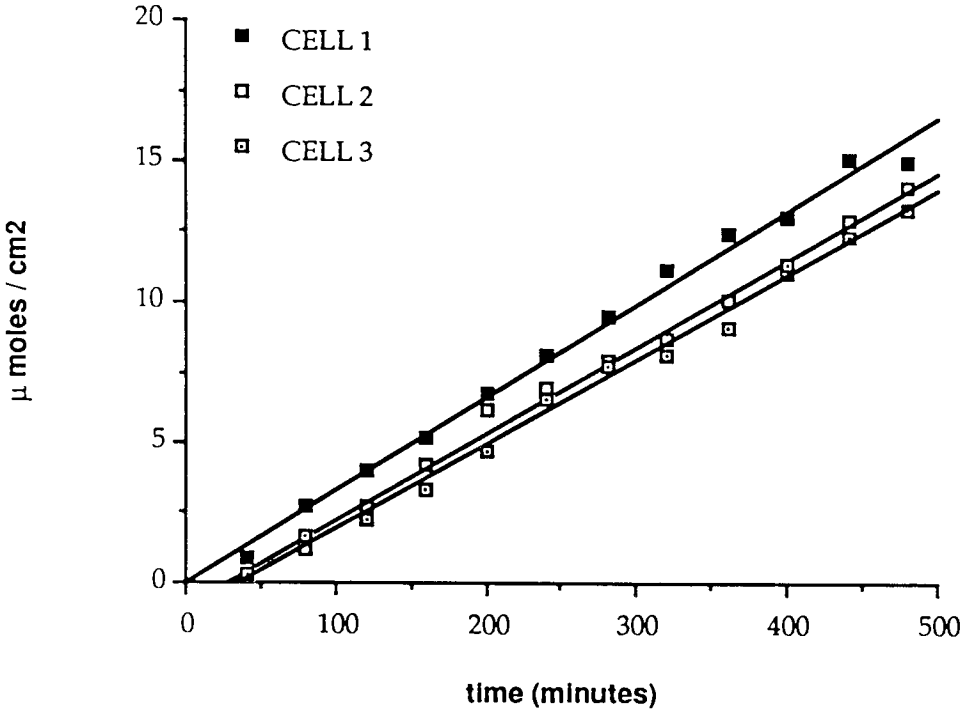
The steady-state fluxes, permeability coefficients and lag times were determined from the linear portions of the rate curves by linear regression as described in section 4.2. The results are summarised in table 4.2.

Table 4.2 Diffusion data for 0.025M tryptophan through the same batch of mucus on consecutive days (1 and 2). The mean value presented is the mean ± sem of the three determinations.

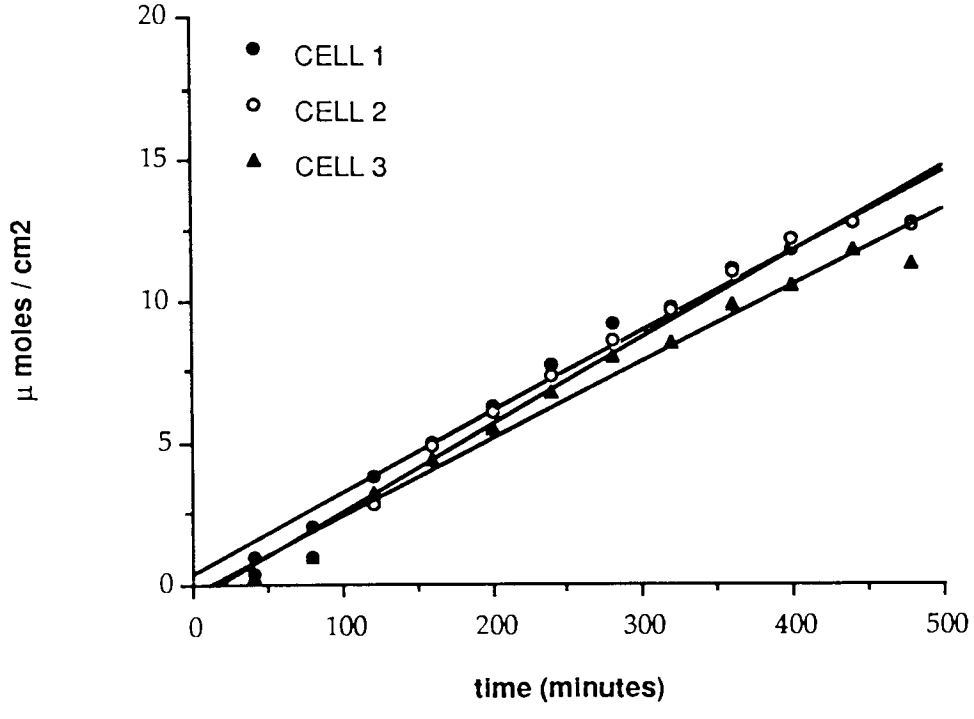
day	cell	flux (x 10^4) μ moles cm^{-2} min^{-1}	K_p (x 10^3) cm min^{-1}	lag time min
1	1	332.3	1.3292	-1.05
1	2	309.1	1.2362	27.22
1	3	300.9	1.2034	36.11
	mean	314.1 ± 9.42	1.2566 ± 0.038	20.76 ± 11.20
2	1	284.2	1.1368	-1.29
2	2	305.4	1.2215	16.55
2	3	269.9	1.0797	9.39
	mean	286.5 ± 10.3	1.146 ± 0.04	8.22 ± 5.11

Figure 4.3 The diffusion of 0.025M tryptophan through the same batch of mucus on a) day 1 and b) day 2. Experiments were performed in phosphate buffer (pH 7.4) at 20°C.

a)



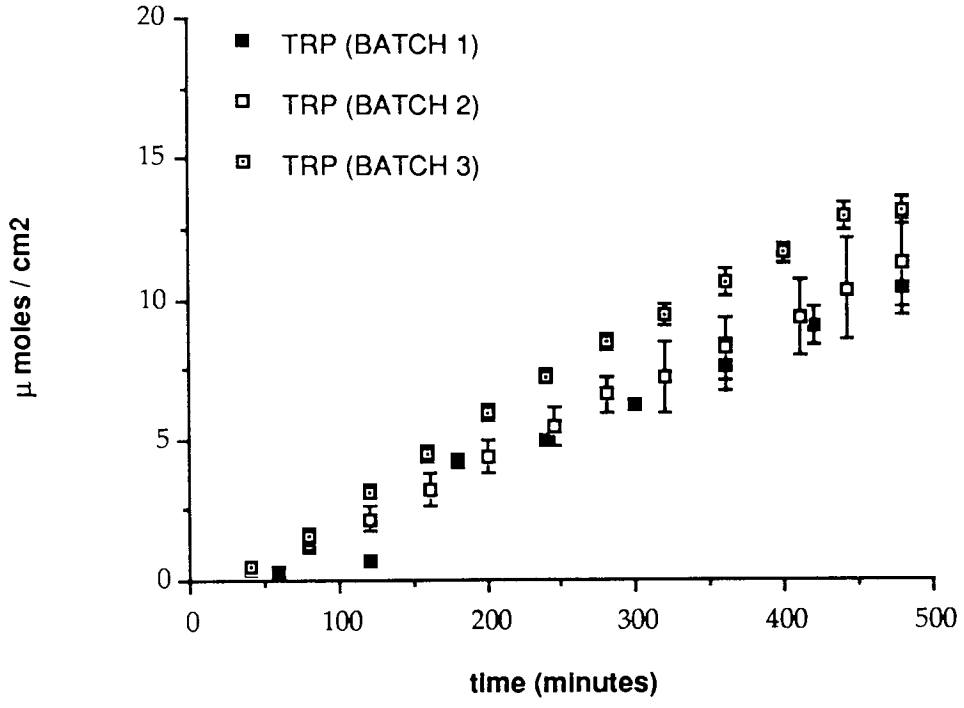
b)



On day 1 the mean flux for the three determinations was 0.031 ± 9.42 x 10^{-4} μ moles cm^{-2} min^{-1} and the lag time 20.76 ± 11.20 min. The mean flux value for the three determinations on day 2 was 0.029 ± 10.3 x 10^{-4} μ moles cm^{-2} min^{-1}, which was slower than that obtained on day 1, but not appreciably different. The mean lag time obtained for day 2 was 8.22 ± 5.11 min, which again was not considerably different to that obtained on day 1.

The results obtained for the diffusion of 0.025M TRP through different batches of mucus are illustrated in figure 4.4.

Figure 4.4 The diffusion of 0.025M tryptophan through three different batches of mucus. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of three determinations.



The steady-state fluxes, permeability coefficients and lag times were determined by linear regression from the initial linear portion of the rate curves. The results are summarised in table 4.3.

Table 4.3 Diffusion data for 0.025M tryptophan through three different batches of mucus. Values are the mean ± sem of at least three determinations.

batch	flux (x 10^4) μ moles cm^{-2} min^{-1}	K_p (x 10^3) cm min^{-1}	lag time min
1	265.4 ± 16.7	1.0612 ± 0.067	12.89 ± 2.96
2	255.8 ± 17.5	1.0232 ± 0.070	31.44 ± 5.74
3	300.3 ± 8.78	1.2012 ± 0.025	39.04 ± 3.81

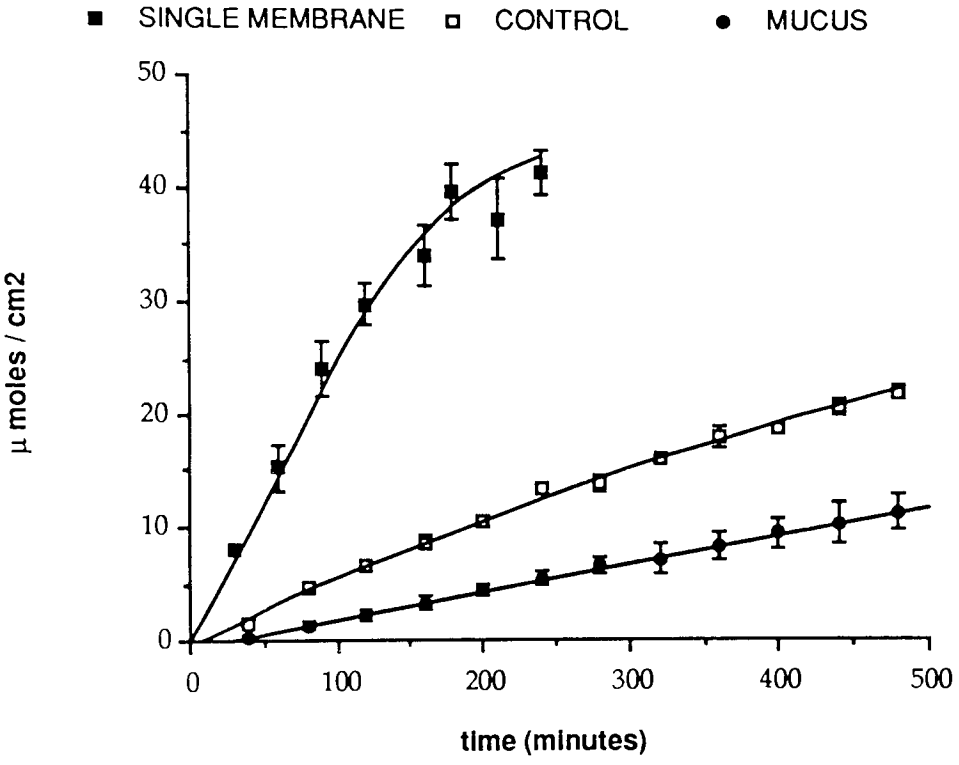
The mean flux values ranged from 255.8 x 10^{-4} μ moles cm^{-2} min^{-1} to 300.3 10^{-4} μ moles cm^{-2} min^{-1} hence with a range of 44.5 x 10^{-4} μ moles cm^{-2} min^{-1}. This range was slightly larger than the range of 35.5 x 10^{-4} μ moles cm^{-2} min^{-1} observed for the mean flux rates through the same batch of mucus (on day 2). The flux rates through different batches were not however markedly different to each other.

4.4.2 The retardation the diffusion of tryptophan by mucus

The retardation of diffusion of 0.025M TRP by mucus was assessed by determining the rate of diffusion through a single Nuclepore membrane positioned between the donor and receiver compartments, through a 1 mm thick unstirred aqueous layer and through a mucus layer of the same thickness.

With a single Nuclepore polycarbonate membrane positioned between the donor and receiver compartment, TRP rapidly diffused across the membrane with a mean flux of (2600 ± 300) x 10^{-4} μ moles cm^{-2} min^{-1} calculated by linear regression of the first five points. Moreover, the concentration in the receiver phase was rapidly approaching the equilibrium value at 180 min. This is illustrated in figure 4.5

Figure 4.5 The diffusion of 0.025M tryptophan across a single Nuclepore polycarbonate membrane (SINGLE MEMBRANE), through an aqueous unstirred layer of 1 mm thickness (CONTROL) and through a mucus layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of at least three determinations.



The imposition of the central compartment between the donor and receiver compartment producing an aqueous unstirred layer of 1 mm reduced the flux of TRP to (520 ± 7.22) x 10^{-4} μ moles cm^{-2} min^{-1}. This corresponded to a 5-fold reduction in flux.

The introduction of a 1 mm thick layer of mucus into the central compartment further reduced the flux of TRP to a value of (283 ± 10.5) x 10^{-4} μ moles cm^{-2} min^{-1}. The value of the mucus retardation ratio calculated by crossover-analysis was 55.12 ± 5.94 %. This is equivalent to a 2-fold reduction in diffusion flux by mucus. A similar 2-fold reduction in diffusion rates by mucus has been reported for hydrogen ion diffusion (Pfeiffer, 1981; Winne and Verheyen, 1990). An increase in the lag times was also observed. The values calculated for the steady-state fluxes, permeability coefficients and lag times are given in table 4.4.

Table 4.4 Diffusion data for 0.025M tryptophan (TRP) across a single Nuclepore polycarbonate membrane using first five points only, through an aqueous unstirred layer of 1 mm thickness (CONTROL) using first seven points only, and through a mucus layer of 1 mm thickness (MUCUS). Values are the mean ± sem of at least three determinations.

TRP	flux (x 10^4) μ moles cm^{-2} min^{-1}	K_p (x 10^3) cm min^{-1}	lag time min
SINGLE	2600 ± 300	10.2 ± 0.62	-1.23 ± 3.83
CONTROL	520 ± 7.22	2.079 ± 0.029	2.35 ± 0.97
MUCUS	283 ± 10.5	1.132 ± 0.042	25.07 ± 1.53

4.4.3 Retardation of diffusion by mucus.

To determine the nature of the mucus layer as a barrier to peptide delivery the influence of molecular weight, charge, lipophilicity and chemical group on the rate of diffusion through mucus were investigated. The rates of diffusion of WG, WGG, BA, PR, OX, AT, NP and ME at 0.025M concentrations through aqueous and mucus layers were determined at 20°C.

The rate of diffusion through mucus for all of the compounds tested was significantly slower ($p < 0.05$) than through an aqueous unstirred layer of the same thickness. Thus, compared with free water, the mucus gel represents a considerable barrier to drug molecules.

In the case of the dipeptide WG, shown in figure 4.6, the flux through the unstirred aqueous layer was (553 ± 15.2) x 10^{-4} μ moles cm^{-2} min^{-1} which was reduced to (312 ± 3.00) x 10^{-4} μ moles cm^{-2} min^{-1} through the mucus layer. This represents a retardation ratio of 56.57 ± 2.51 %. The lag time through the aqueous layer was 9.17 ± 8.43 min which was increased to 33.06 ± 12.54 min by the introduction of the mucus layer, which was significantly longer ($p < 0.05$). The numerical diffusion data for WG is presented in table 4.5.

Figure 4.6 The diffusion of 0.025M tryptophylglycine through an aqueous unstirred layer of 1 mm thickness (CONTROL) and through a mucus layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of at least three determinations.

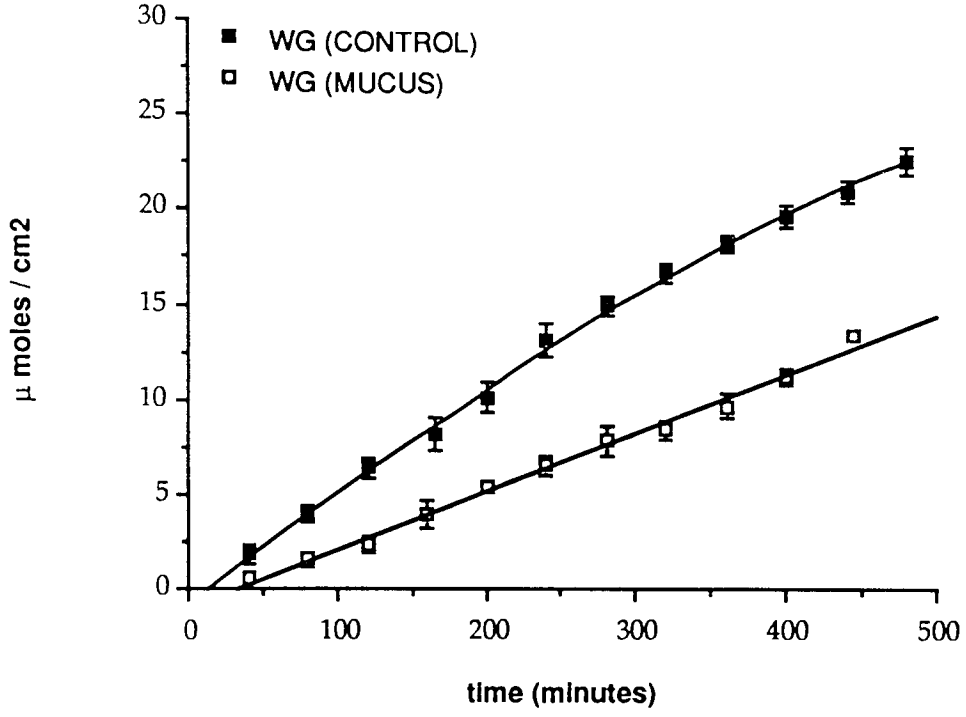


Table 4.5 Diffusion data for 0.025M tryptophylglycine (WG) through an aqueous unstirred layer of 1 mm thickness (CONTROL) using the first seven points only and through a mucus layer of 1 mm thickness (MUCUS). Values are the mean ± sem of at least three determinations.

WG	flux (x 10^4) μ moles cm^{-2} min^{-1}	K_p (x 10^3) cm min^{-1}	lag time min
CONTROL	553 ± 15.2	2.212 ± 0.608	9.17 ± 8.43
MUCUS	312 ± 3.00	1.250 ± 0.012	33.06 ± 12.54

The diffusion of the tripeptide WGG is illustrated in figure 4.7. The rate of diffusion through the aqueous unstirred layer was (461 ± 84.3) x 10^{-4} μ moles cm^{-2} min^{-1} which was reduced to (221 ± 8.02) x 10^{-4} by the mucus layer. This corresponds to a mucus retardation ratio of 40.94 ± 3.80 %. The numerical diffusion data for WGG is given in table 4.6.

Figure 4.7 The diffusion of 0.025M tryptophylglycylglycine through an aqueous unstirred layer of 1 mm thickness (CONTROL) and through a mucus layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of at least three determinations.

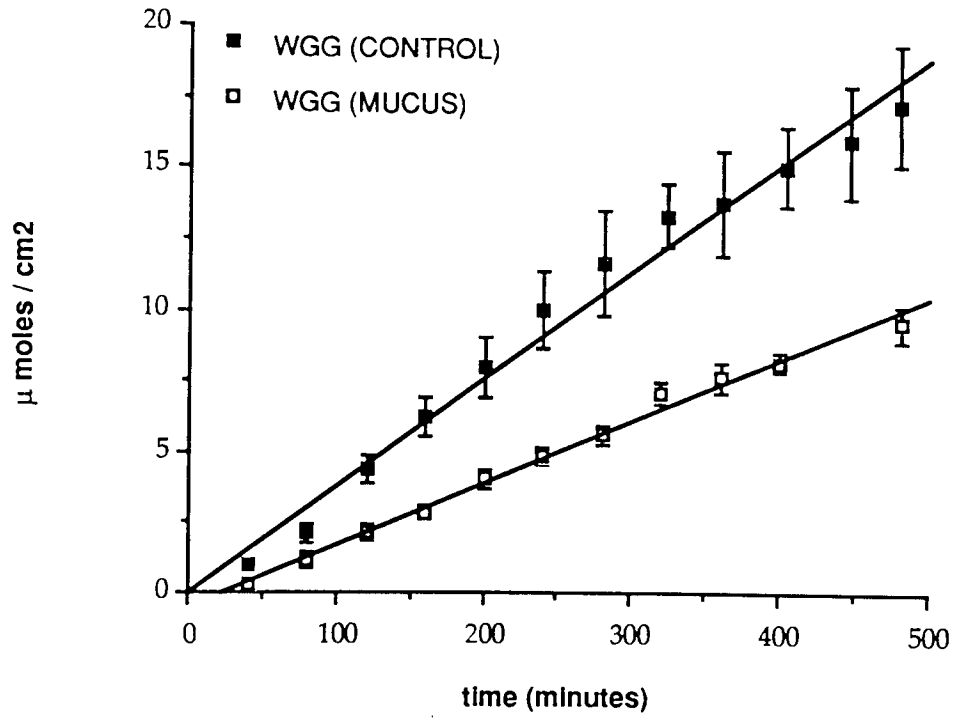


Table 4.6 Diffusion data for 0.025M tryptophylglycylglycine (WGG) through an aqueous unstirred layer of 1 mm thickness (CONTROL) using the first seven points only, and through a mucus layer of 1 mm thickness (MUCUS). Values are the mean ± sem of at least three determinations.

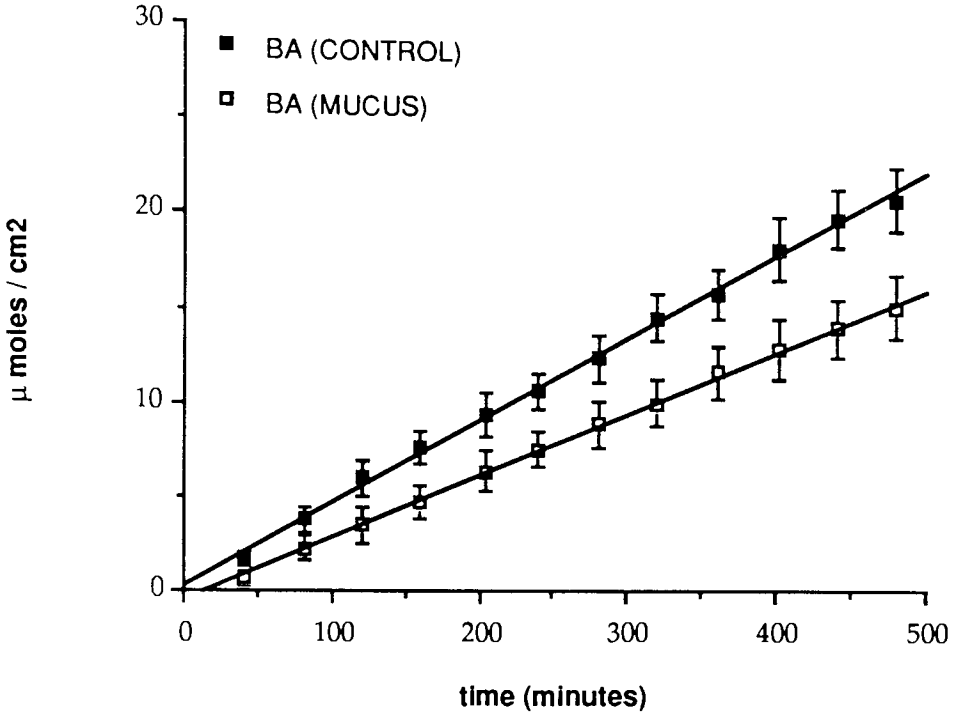
WGG	flux (x 10^4) μ moles cm^{-2} min^{-1}	K_p (x 10^3) cm min^{-1}	lag time min
CONTROL	461 ± 84.3	1.844 ± 0.337	21.34 ± 11.28
MUCUS	221 ± 8.02	0.882 ± 0.032	23.16 ± 4.29

The diffusion of the carboxylic acid BA through aqueous control and mucus layers is illustrated in figure 4.8. For this compound the flux through the aqueous control was (438 ± 30) x 10^{-4} μ moles cm^{-2} min^{-1}, which was reduced to (330 ± 10.6) x 10^{-4} μ moles cm^{-2} min^{-1} by the mucus layer. The mucus retardation ratio for this compound was 76.17 ± 9.18 %. The numerical diffusion data for this compound is given in table 4.7.

Table 4.7 Diffusion data for 0.025M benzoic acid (BA) through an aqueous unstirred layer of 1 mm thickness (CONTROL) and through a mucus layer of 1 mm thickness (MUCUS). Values are the mean ± sem of at least three determinations.

BA	flux (x 10^4) μ moles cm^{-2} min^{-1}	K_p (x 10^3) cm min^{-1}	lag time min
CONTROL	438 ± 30.0	1.752 ± 0.120	-6.16 ± 11.23
MUCUS	330 ± 10.6	1.321 ± 0.042	13.98 ± 9.62

Figure 4.8 The diffusion of 0.025M benzoic acid through an aqueous unstirred layer of 1 mm thickness (CONTROL) and through a mucus layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem od at least three determinations.



The diffusion of the three β-blockers PR, OX and AT through the mucus layer was in each case significantly slower ($p < 0.05$) than through an aqueous layer of the same thickness. The graphical results for PR, OX and AT are illustrated in figures 4.9, 4.10 and 4.11 respectively. The numerical diffusion data for these three compounds are presented in tables 4.8, 4.9 and 4.10. The mucus retardation ratios were calculated to be 55.50 ± 5.65%, 51.58 ± 2.91% and 46.32 ± 5.84% for PR, OX and AT respectively.

Figure 4.9 The diffusion of 0.025M propranolol through an aqueous unstirred layer of 1 mm thickness (CONTROL) and through a mucus layer of 1 mm thickness, using two different batches of mucus (MUCUS1) and (MUCUS2). Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of at least three determinations.

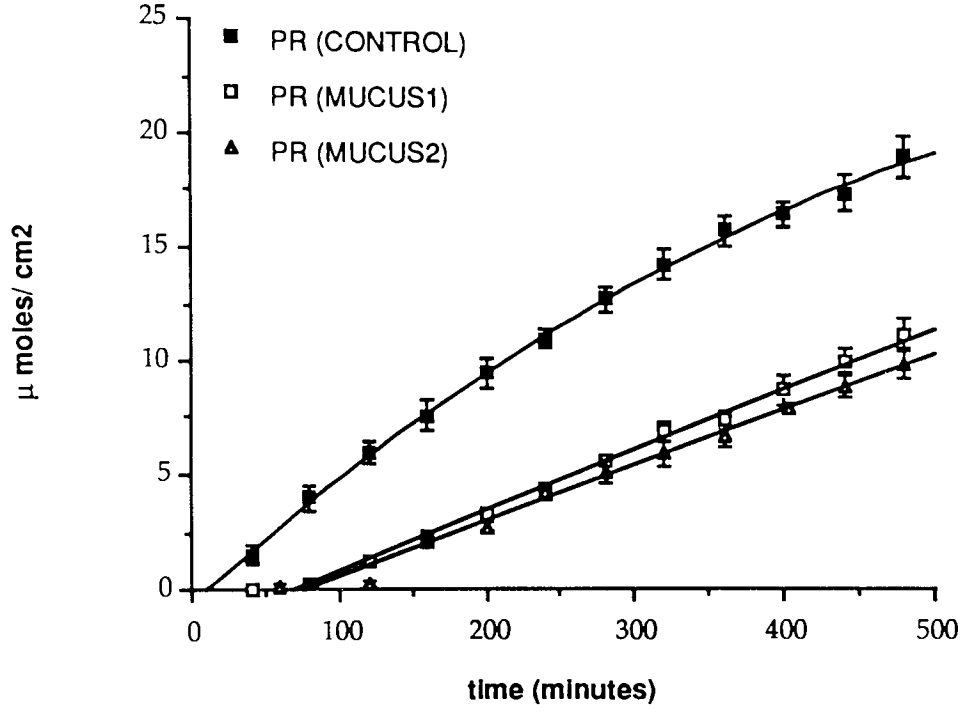


Table 4.8 Diffusion data for 0.025M propranolol (PR) through an aqueous unstirred layer of 1 mm thickness (CONTROL) using the first seven points only, and through a mucus layer of 1 mm thickness (MUCUS). Values are the mean ± sem of at least three determinations.

PR	flux (x 10^4) μ moles cm^{-2} min^{-1}	K_p (x 10^3) cm min^{-1}	lag time min
CONTROL	459 ± 15.1	1.833 ± 0.061	-3.02 ± 10.79
MUCUS	254 ± 9.92	1.016 ± 0.040	72.25 ± 3.64

Figure 4.10 The diffusion of 0.025M oxprenolol through an aqueous unstirred layer of 1 mm thickness (CONTROL) and through a mucus layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of at least three determinations.

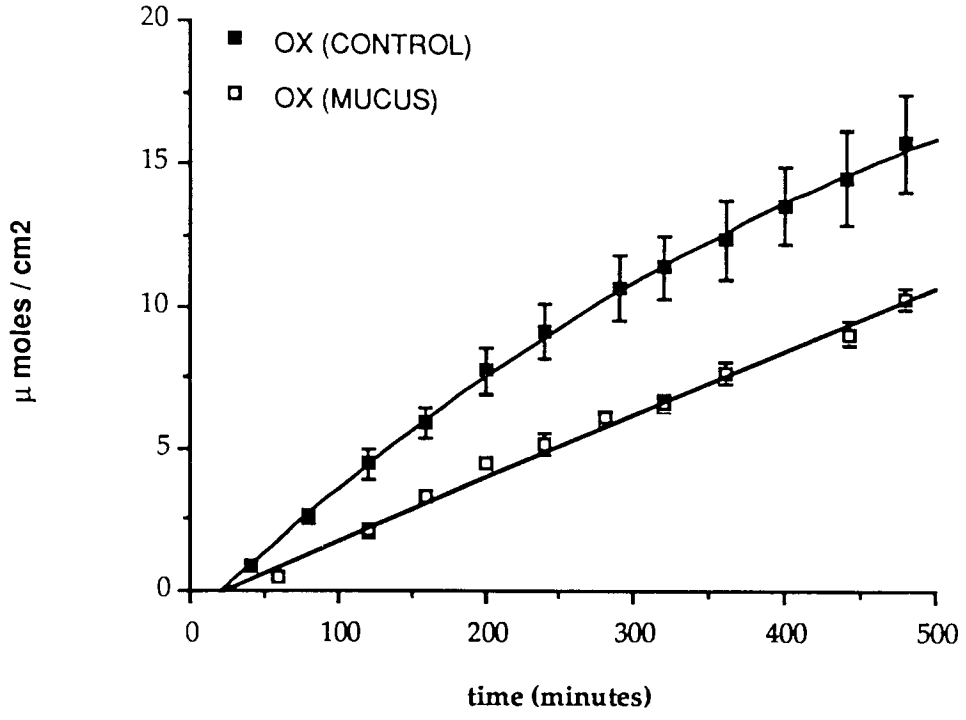


Table 4.9 Diffusion data for 0.025M oxprenolol (OX) through an aqueous unstirred layer of 1 mm thickness (CONTROL) using the first seven points only, and through a mucus layer of 1 mm thickness (MUCUS). Values are the mean ± sem of at least three determinations.

OX	flux (x 10^4) µ moles cm^{-2} min^{-1}	K_p (x 10^3) cm min^{-1}	lag time min
CONTROL	398 ± 49.4	1.593 ± 0.198	11.11 ± 7.51
MUCUS	225 ± 6.57	0.899 ± 0.026	19.83 ± 5.37

Figure 4.11 The diffusion of 0.025M atenolol through an aqueous unstirred layer of 1 mm thickness (CONTROL) and through a mucus layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of at least three determinations.

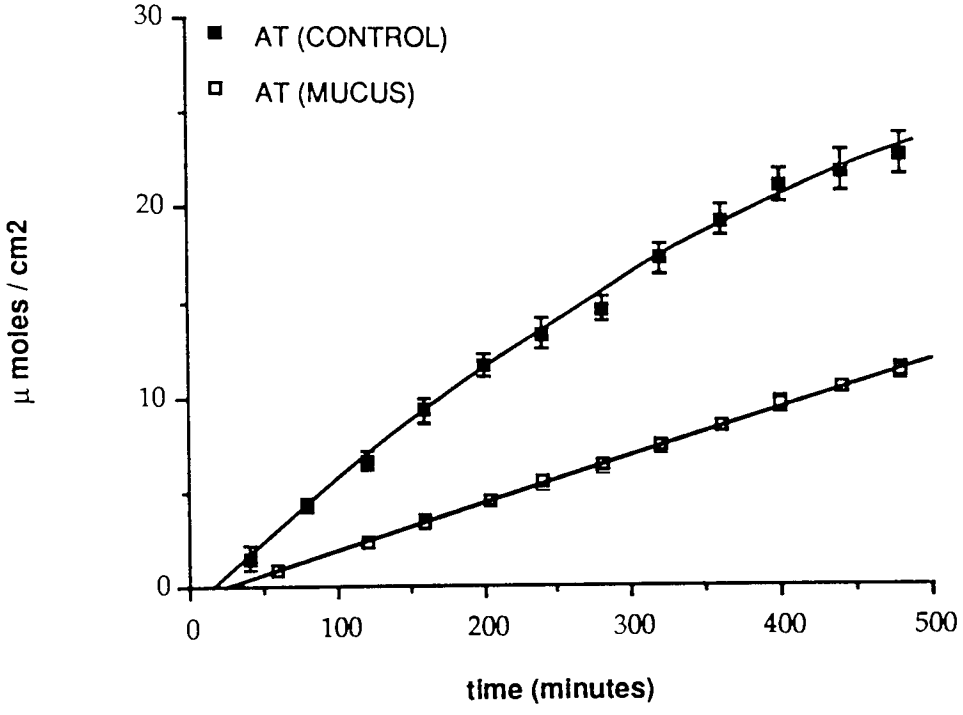


Table 4.10 Diffusion data for 0.025M atenolol (AT) through an aqueous unstirred layer of 1 mm thickness (CONTROL) using the first seven points only, and through a mucus layer of 1 mm thickness (MUCUS). Values are the mean ± sem of at least three determinations.

AT	flux (x 10^4) µ moles cm^{-2} min^{-1}	K_p (x 10^3) cm min^{-1}	lag time min
CONTROL	551 ± 26.6	2.202 ± 0.106	-0.254 ± 6.91
MUCUS	253 ± 4.61	1.013 ± 0.018	25.88 ± 10.24

The diffusion of the N-amide of phenylalanine, NP, through mucus an aqueous layer is illustrated in figure 4.12. The flux through the aqueous control was (543 ± 1.24) x 10^{-4} μ moles cm^{-2} min^{-1} which was reduced to (253 ± 31.0) x 10^{-4} μ moles cm^{-2} min^{-1} by the mucus layer. The numerical diffusion data for this compound are presented in table 4.11. The mucus retardation value for NP was calculated to be 46.51 ± 8.85%.

Figure 4.12 The diffusion of 0.025M N-acetylphenylalanine through an aqueous unstirred layer of 1 mm thickness (CONTROL) and through a mucus layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of at least three determinations.

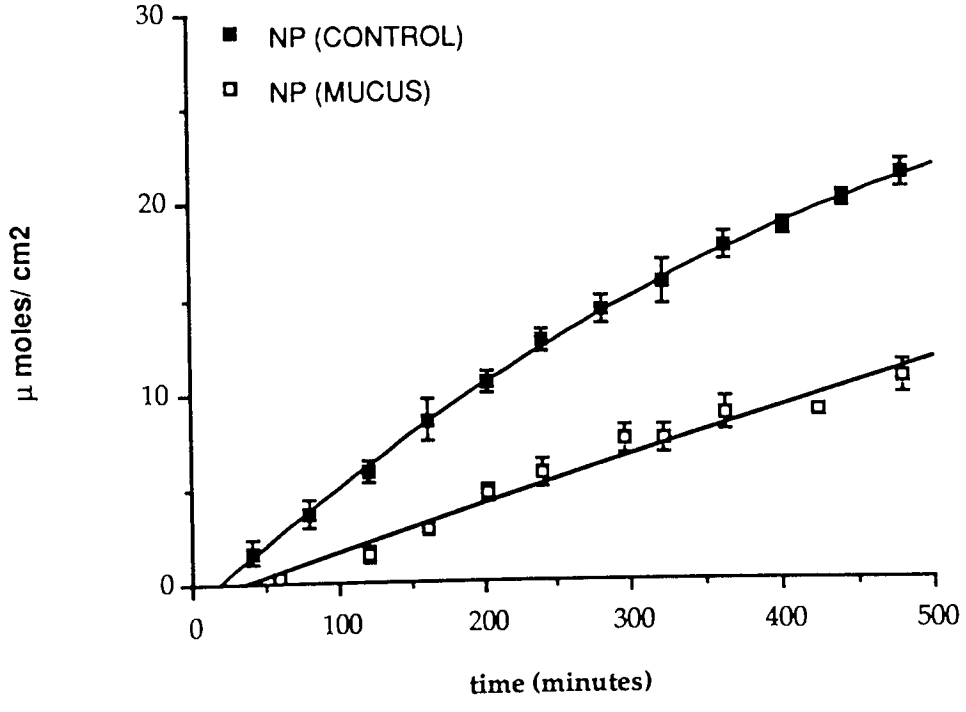


Table 4.11 Diffusion data for 0.025M N-acetylphenylalanine (NP) through an aqueous unstirred layer of 1 mm thickness (CONTROL) using the first seven points only, and through a mucus layer of 1 mm thickness (MUCUS). Values are the mean ± sem of at least three determinations.

NP	flux (x 10^4) μ moles cm^{-2} min^{-1}	K_p (x 10^3) cm min^{-1}	lag time min
CONTROL	543 ± 1.24	2.17 ± 5 x 10^{-3}	10.01 ± 8.03
MUCUS	253 ± 31.0	1.010 ± 0.124	33.51 ± 13.33

The diffusion of ME through an aqueous control was carried out prior to evaluating its rate of diffusion through the mucus gel and compared with the diffusion rates obtained for PHE and NP through an aqueous layer. These results are illustrated in figure 4.13. In the case of the amino acid PHE the diffusion curve was linear. Similarly, the diffusion curve for NP was initially linear but was exhibiting curvature in the latter stages of its profile. The diffusion profile of the methyl ester, ME however, in contrast to the other phenylalanine based compounds began to plateau at 240 min and concentrations in the receiver phase did not increase thereafter.

This unexpected result, with the graph plateauing at concentrations well below the equilibrium concentration implied that the compound ME was being removed from the system by some mechanism. To investigate whether this phenomena was due to the degradation of ME to PHE, 0.025M ME was incubated under the same experimental conditions as in the diffusion experiments, i.e. in phosphate buffer at 20°C for 10 hr. At set times samples were removed and the concentration of either ME or PHE determined. The results of this experiment are illustrated in figure 4.14 and demonstrate that ME was hydrolysed to the aromatic amino acid phenylalanine at such a rate that at 8 h approximately 70 % of the ME remained intact.

Figure 4.13 The diffusion of 0.025M N-acetylphenylalanine (NP), phenylalanine (PHE) and the methyl ester of phenylalanine (ME) through an aqueous unstirred layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of at least three determinations.

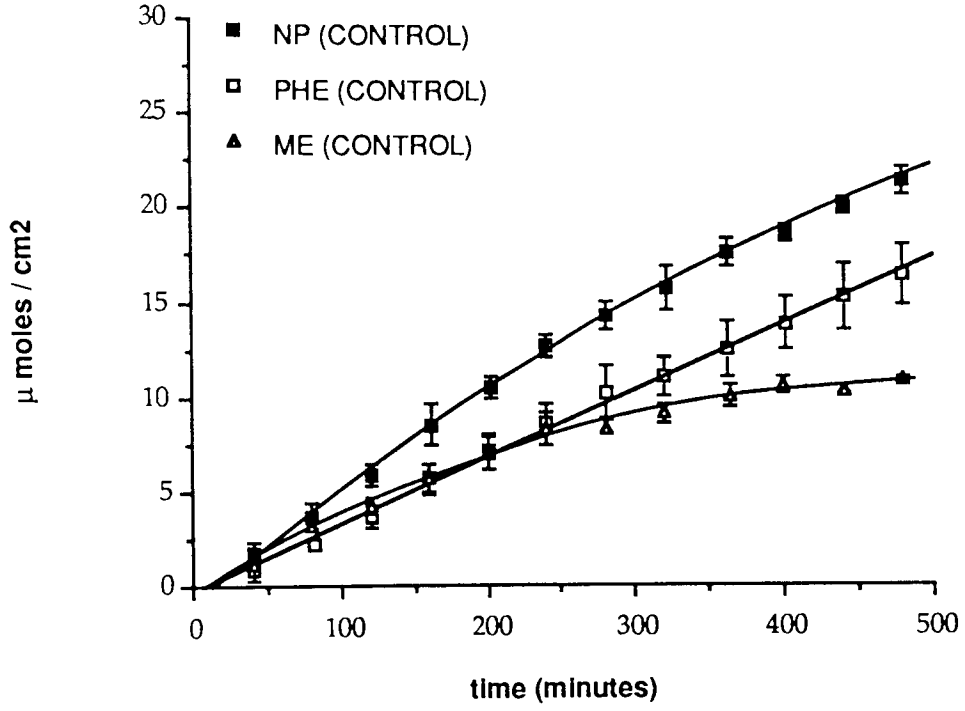
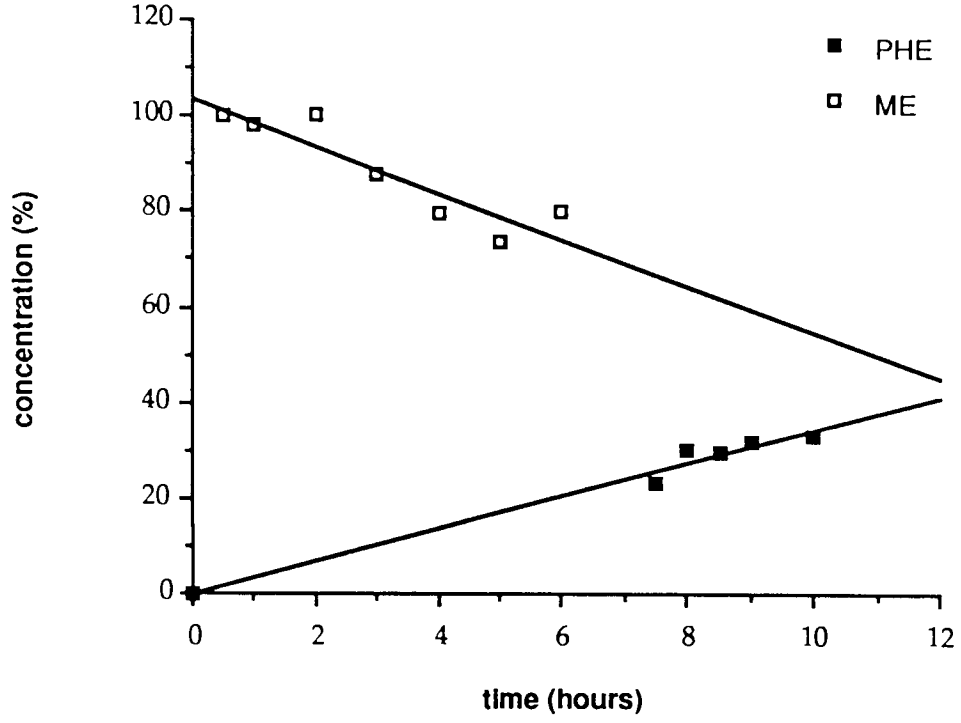


Figure 4.14 The hydrolysis of the methyl ester of phenylalanine (ME) to phenylalanine (PHE) in phosphate buffer (pH 7.4) at 20°C. Initial concentration of ME was 0.025M. Points are for a single determination.



In a further attempt to investigate the effect of blocking the carboxyl group of an amino acid on the rate of diffusion through mucus the compound phenylalaninol was selected. This compound possessed an alcohol group in place of a carboxyl group whilst retaining the free amino group. Analytical development was carried out for this compound and its diffusion through an aqueous unstirred layer determined. It was difficult to obtain meaningful results for this compound as from observation it was found that the compound rapidly reacted with the stationary phase of the analytical HPLC column rendering the calibration curves meaningless and ultimately destroying the column. For these reasons the investigation of the diffusion of this compound through mucus was abandoned.

Clearly, from the diffusion results obtained for all evaluated compounds, the mucus gel is a significantly greater barrier to drug diffusion than a free water layer. This is in contrast to the view of Heatley (1959), where there was no observed difference between diffusion through the two layers. The diffusion results for all eight compound are summarised in figure 4.15 as the mucus retardation ratios. The numerical values of the mucus retardation ratios are presented in table 4.12. The pathway for diffusion through the mucus gel (as all other gels) will be through the fluid phase entrapped within the gel (Barry, 1983). In the case of mucus the fluid phase will be water, which constitutes 90-95 % of the gel by weight (Creeth, 1978; Carlstedt et al., 1985).

The factors that affect diffusion through free water may then be applicable to diffusion though mucus. An important factor which affects the rate of diffusion through a free liquid is the diffusant concentration, which is usually taken as the driving force for diffusion. The frictional resistance to diffusion is also important and is dependent mainly upon the particle size and shape in addition to the type of solvent. Interactions between the diffusing drug molecules such as self-aggregation and micellisation, which may alter the particle size, shape and the thermodynamic activity coefficient will similarly exert an effect on the diffusion rate. Finally,

Figure 4.15 The retardation of diffusion by mucus of all compounds tested expressed as a percentage of the rate of diffusion through 1 mm mucus layer/ rate of diffusion through an aqueous unstirred layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± standard deviation.

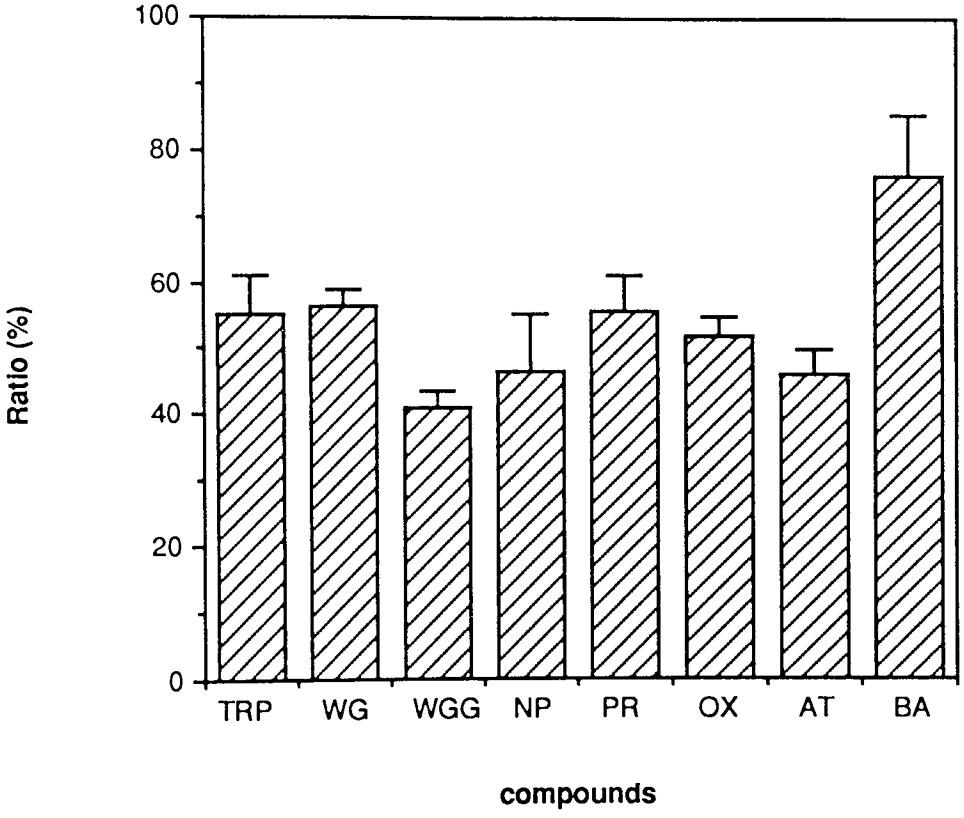


Table 4.12 Diffusion data for mucus retardation ratios of all eight compounds tested. Values are the mean ± standard deviation of nine determinations.

Compound	mucus retardation ratio (%)
TRP	55.12 ± 5.94
WG	56.57 ± 2.51
WGG	40.94 ± 3.80
NP	46.51 ± 8.85
PR	55.50 ± 5.65
OX	51.58 ± 2.91
AT	46.23 ± 3.67
BA	76.16 ± 9.18

temperature variations will alter the diffusion rate by imparting increased thermal motion to the diffusing solute and by altering the viscosity of the solvent (Barry, 1983). A theoretical general formula has been proposed by Peppas et al. (1984) which describes the expected effect of solute size on diffusion through mucus.

In the studies described in this section equimolar concentration donor solutions were used and all the experiments were performed at 20°C. Any differences in the rates of diffusion will thus be due to other factors, which will be discussed here.

The first question to ask is "How does the mucus layer retard the rate of diffusion?". The mucus layer may be modifying the observed diffusivity in an non-selective manner by a mechanical obstructive effect. Alternatively, it may present a more selective barrier whereby the rate of diffusion will be determined by the chemical nature of the diffusing molecule, due to chemical interactions between the molecules comprising the mucus gel and the diffusant. The retardation may also be a result of a combination of both these mechanisms.

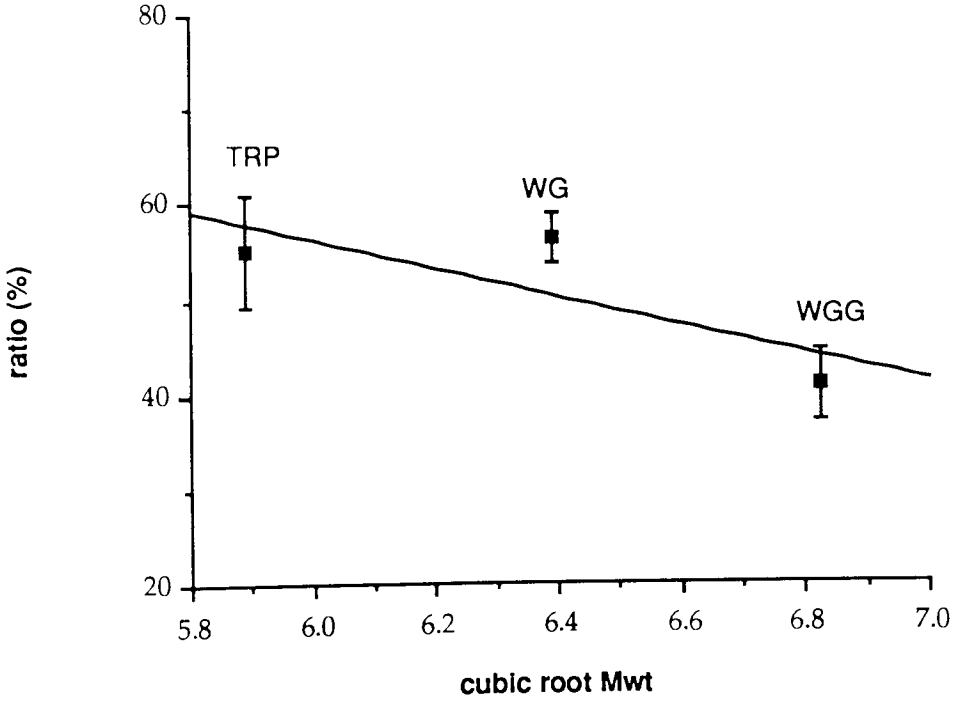
The mechanical obstructive barrier is most easily understood. The presence of the gel simply prevents the free flow transfer as would a piece of filter paper. This would be achieved by reducing the open area available for diffusion and increasing the actual path length of diffusion due to increased path tortuosity through the gel structure. It has also been postulated that there is a change in the structure of water immobilised within a gel (Williams and Turnberg, 1981). This water is believed to be highly ordered as a result of tight H_2O-H_2O bonding and consequently within the gel there are zones of highly viscous water which are responsible for the observed retardation in diffusion. Lee and Nicholls (1987) have suggested that at the very most only 5 % of the water within the mucus gel is likely to be affected by such a phenomenon, which implies that the remaining water within the gel possesses the bulk properties of free water.

If mechanical obstruction were predominant in retarding the rate of diffusion the phenomena would be dependent upon the size and shape of the solute molecule in addition to the volume fraction of the polymer. As the diffusant traversed the gel it would collide with individual molecules which comprise the mucus gel and the rate of transport would be reduced. Clearly, as the size of the diffusant increases the probability of it colliding with a polymer molecule also increases. The observed result would be that the rate of diffusion of larger molecules through mucus would be slower than smaller molecules.

In this study the compounds TRP, WG and WGG were chosen to investigate the effect of increasing molecular weight which possess molecular weights of 204.23, 261.3 and 318.3g respectively. Thus they represent a chemically similar series with a molecular weight range of 114.07 Da. The result of plotting the cubic root of the molecular weight against the mucus retardation ratio for these three compounds is shown in figure 4.16. Linear regression indicated a linear trend, with increasing molecular weight reducing the transport rate through mucus. Increasing the molecular weight from by 114.07, significantly reduced the mucus retardation ratio from 55.12 ± 5.94% in the case of TRP to

40.94 ± 3.80% for WGG ($p < 0.05$). The determined value for WG (56.57 ± 2.51 %) was larger than TRP, although the difference was not significant ($p < 0.05$).

Figure 4.16 The effect of increasing molecular weight on the rate of diffusion of tryptophan (TRP), tryptophylglycine (WG) and tryptophylglycylglycine (WGG) through mucus. The retardation of diffusion by mucus is expressed as a percentage of the rate of diffusion through 1 mm mucus layer/ rate of diffusion through an aqueous unstirred layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± standard deviation.



It is appreciated that the molecular weight range employed is rather small, particularly in view of the fact that a large number of peptidic drugs are at least decapeptides and have correspondingly high molecular weights, exceeding 318. It was initially proposed to investigate the effect of molecular weight with respect to peptide diffusion through mucus with the amino acid phenylalanine, the dipeptide FF, tripeptide FFF up to the pentapeptide FFFFF. The aqueous solubilities of FF and FFF were however negligible and it was impossible to prepare solutions of these compounds which could diffuse through the mucus layer and produce quantifiable amounts in the receiver compartment. The use of cosolvents, such as DMSO, could increase the solubility slightly but to produce a 1 mM solution of FFF required 20 % DMSO. In view of the fact that solvents such as ethanol are known to alter the observed diffusion rates through mucus (Turner et al., 1985) this methodology was abandoned.

The diffusion of a peptide series based on the small readily water soluble amino acid glycine was explored but not experimentally carried out due to the analytical problems encountered in separating these very similar molecules from each other, which was thought necessary for preliminary studies to ensure the intact molecule was penetrating the mucus gel, but more importantly from the solvent front. The compounds were glycine, GG, GGG, GGGG and GGGGG. Another considerable problem was achieving the necessary sensitivity to detect the diffusants in the receiver phase as none of these molecules contained an aromatic residue and at 210 nm there was considerable background absorption from both the buffer and from components leaching out from the mucus during the course of the experiment. A assay was determined using an amino acid analyser, employing ion-exchange and ninhydrin derivatisation to resolve G and GG. The

analysis time to achieve an adequate resolution of G and GG, was in excess of 2 h, which was considered unusable in view of the large number of samples, which would need to be analysed in the diffusion experiments.

The result of comparing the cubic root of the molecular weight against the mucus retardation ratio for all eight compounds is illustrated in figure 4.17.

Figure 4.17 The effect of increasing molecular weight on the rate of diffusion of all the compounds tested through mucus. The retardation of diffusion by mucus is expressed as a percentage of the rate of diffusion through 1 mm mucus layer/ rate of diffusion through an aqueous unstirred layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± standard deviation.

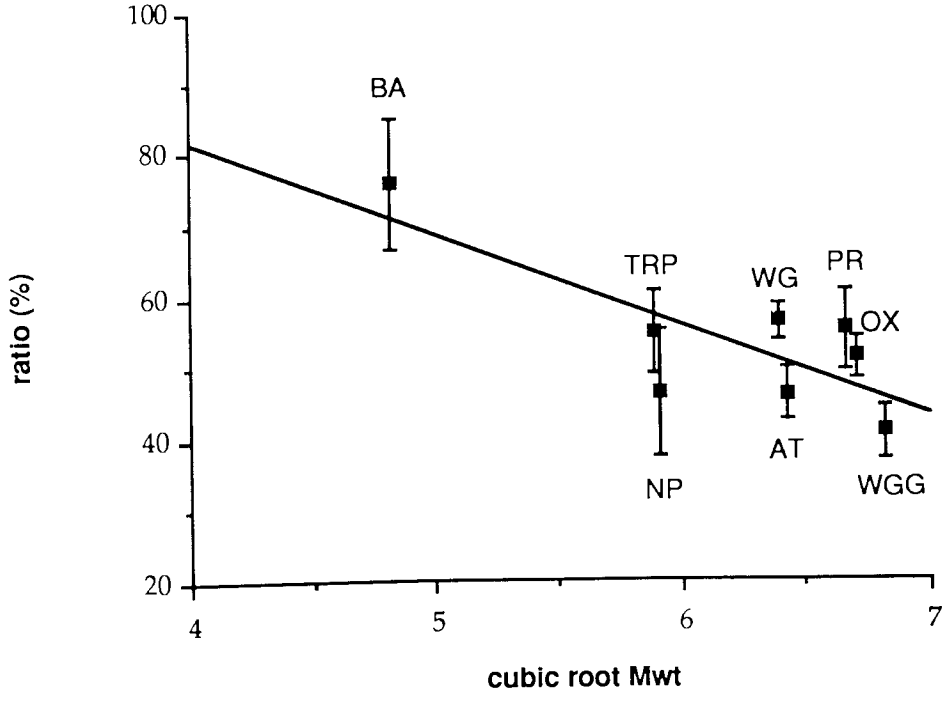


Figure 4.17 indicated a linear trend between the cubic root of molecular weight and the mucus retardation ratio, as seen for the series TRP, WG and WGG. It suggested that increasing molecular weight led to a reduction in transport through mucus.

This reduction in diffusion rate with increasing molecular weight has also been observed by Hughes (1988) with a series of eight chemically different compounds. A statistically significant correlation between the diffusion coefficient and the cubic route of the molecular weight was observed. Saggers and Lawson (1966) using artificial hog gastric mucin and a series of eighteen antibiotics observed an opposite trend between the rate of diffusion through mucus and molecular weight, but no statistical validation was performed. Their observation was that higher molecular weight antibiotics diffused through the 15 % hog mucin column more rapidly than those with low molecular weights and concluded that some form of gel filtration, by size exclusion was occurring. The molecular weight range was from 356 to 960.

It should be noted however, that antibiotics of very different chemical structures were employed in this study, which may have influenced the rates of diffusion. Additionally, artificial hog gastric mucin was employed, which has very different physicochemical properties to native mucus. Perhaps most significantly in this study was the contradiction to this conclusion, that three of the highest molecular weight compounds tested: neomycin (Mwt = 598); paromomycin (Mwt = 645) and the polypeptide antibiotic polymyxin B (Mwt = 1176)) were undetectable in the receiver vessel after 24 hr so did not penetrate the column of hog gastric mucin in significant quantities.

Two studies which have investigated the penetration of mucus by high molecular weight species have been reported. Henry et al. (1990) used fluorescent microscopy and image analysis to monitor the diffusion of fluorescein (FITC) labelled dextran (relative molecular mass 35,600) into unpurified pig small intestine mucus. The experimental results indicated that linear molecules of high molecular mass could penetrate a mucus gel of physiological dimensions. No control experiments had

however been carried out to ensure the FITC label remained attached to the dextran for the duration of the experiment.

A study by Radomsky et al. (1991) used a similar technique to study the diffusion of antibodies through diluted cervical mucus. The conclusion of this work was that mucus was not a significant barrier, but the mucus concentrations were not physiological and again the integrity of the label-antibody bond had not been determined.

The observed dependence of retardation of diffusion on molecular weight, support the view that mucus exerts a mechanical obstructive effect and that for higher molecular weight peptidic molecules diffusion through the mucus layer may be a significant barrier. This would be in line with the idea proposed by Edwards (1978) that one of the prime roles of mucus might be to selectively exclude macromolecules and larger particles from contact with cell membranes.

The mechanism by which mucus retards diffusion may not rely purely on a mechanical obstructive effect. Additionally, solute molecules may become adsorbed on to molecules comprising the mucus gel to differing degrees, depending upon their chemical properties.

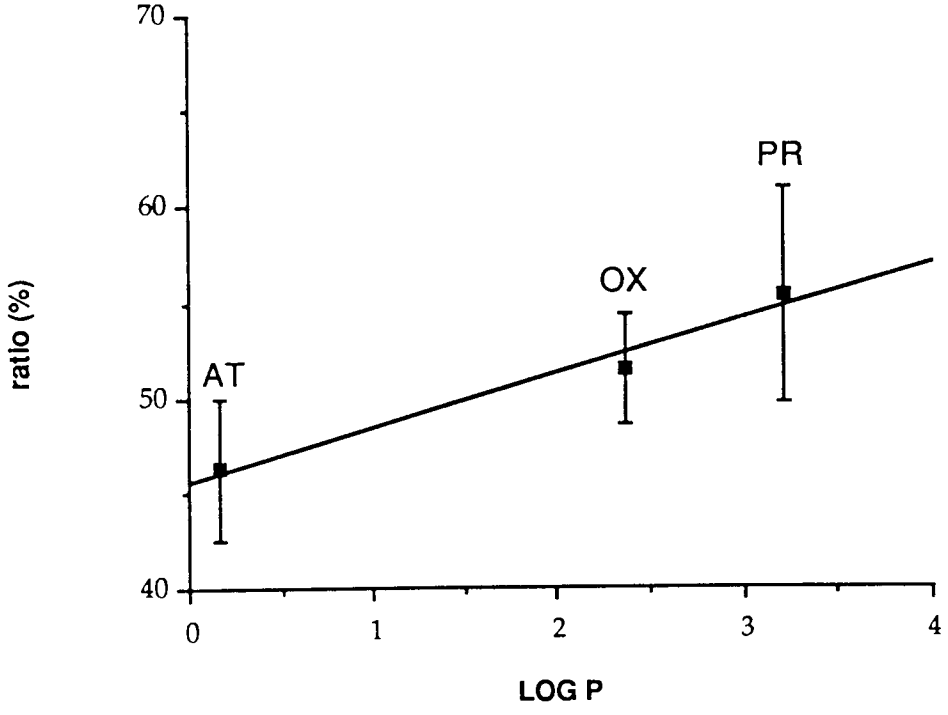
Experiments were carried out in this study to investigate the effect of charge upon the rate of diffusion through mucus using the negatively charged carboxylic acid BA and three positively charged β-blockers PR, OX and AT. The mucus retardation ratio for BA was 76.16 ± 9.18 %, which was the highest value of all eight compounds evaluated, this illustrated that the diffusion of this molecule was least affected by the mucus gel. In contrast, the mucus retardation ratios for the three positively charged β-blockers were significantly slower ($p < 0.05$) being 55.50 ± 5.65, 51.58 ± 5.65 and 46.23 ± 3.67 for PR, OX and AT respectively. The results suggest that the diffusion of a negatively charged molecule through a mucus gel may be less hindered than the transport of one possessing a positive charge.

A similar result with negatively charged compounds diffusing through mucus more rapidly than positively charged molecules was observed by Hughes (1988) who proposed that the negatively charged compound experienced electrostatic repulsion by the mucus gel and so did not interact with the glycoprotein molecules and hence diffused more quickly. Desai and Vadgama, (1991) have also reported the reduced transfer of cationic compounds through native porcine mucus and described the mucus gel as behaving as a weak cation exchange column. This hypothesis has also been proposed by Allen (1978).

This result is perhaps not surprising in view of the large number of charged functional groups within the mucus network. These include negatively charged carboxyl, sulphate and neuraminic acid groups as well as positively charged amino groups located on amino acids within the polypeptide chains of the glycoprotein molecules. At pH 7.4 the neuraminic acid groups are fully dissociated (pKa = 2.6) and the mucus has a resultant net negative charge. The impact of this net negative charge on the rate of diffusion has been investigated directly by Lee and Nicholls (1987) who compared the diffusion of K^+ through pig gastric mucus and pig gastric mucus treated with the enzyme N-acetylneuraminidase to remove neuraminic acid and its associated negative charge. The treatment removed 58 % of the sialic acid and increased the diffusion coefficient significantly from 0.86 ± 0.04 to 1.02 ± 0.06 showing clearly that negatively charged sialic acid groups participate in retarding diffusion through mucus.

To investigate whether compounds with different lipophilicities diffused through mucus at significantly different rates the mucus retardation ratios of PR, OX and AT were plotted against their log P values. This result is illustrated in figure 4.18.

Figure 4.18 The effect of lipophilicity on the rate of diffusion of propranolol, oxprenolol and atenolol through mucus. The retardation of diffusion by mucus is expressed as a percentage of the rate of diffusion through 1 mm mucus layer/ rate of diffusion through an aqueous unstirred layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± standard deviation.



Linear regression analysis was applied to the data, illustrated in figure 4.18 and the following equation obtained:

$$y = 45.53 + 2.91.\log P \qquad (r = 0.986) \qquad (4.21)$$

The result suggested that the more lipophilic compounds, such as PR would diffuse through mucus more rapidly than a similar molecular

weight hydrophilic compound. The mucus retardation ratio of 55.50 ± 5.65 % for the very lipophilic PR was significantly different to the value of 46.23 ± 3.67 % obtained for the hydrophilic AT ($p < 0.05$). This result was unexpected in view of the fluid phase comprising the mucus gel being water.

All of these diffusion experiments were conducted with equimolar donor solutions at the same pH. A further variable which may influence the observed trend in the rates is the varying thermodynamic activity of each compound. The three β-blockers tested, while possessing similar molecular weights and pKa values, have very different lipophilicity values. Consequently, the compounds will have differing solubilities in phosphate buffer. Hence the most lipophilic compound of the three, PR, will be the least soluble, nearer to saturation and have the greatest thermodynamic activity. One requirement for maximum flux is that the drug solution is saturated. Therefore, this trend may simply reflect the differing thermodynamic activities of the compounds in phosphate buffer rather than an effect dependent upon lipophilicity. To investigate this phenomenon the experiments would need to be repeated with drug-saturated solutions.

It was expected that, in view of the above observation demonstrating that positively charged species were particularly retarded by mucus, that blocking the positively charged amino group of PHE to produce the amide NP would increase the rate of diffusion through mucus. The mucus retardation ratio for NP was however calculated to be 46.51 ± 8.85 %, which was significantly lower ($p < 0.05$) than the value of 55.12 ± 5.94 % obtained for TRP, which possessed both a free amino and carboxyl group. This may reflect other factors which are responsible for the retardation of diffusion by mucus. Other known factors which may influence the flux through mucus are steric factors which would hinder entry to pores within the gel and frictional resistance within the pores. Additionally, there may be interactions between the solute and non-glycoprotein molecules such as fatty acids. There are two publications which demonstrate the importance of associated lipids

within the mucus gel on solute diffusivity (Sarosiek et al., 1984; Slomiany et al., 1988)

4.4 SUMMARY

A mucus layer has been shown to provide a considerably larger barrier to the diffusion of drug molecules than an aqueous unstirred layer of the same thickness. The rates of diffusion through mucus were significantly lower than the flux through an aqueous unstirred layer of the same thickness for all eight compounds tested.

The evidence presented here suggests that the observed retardation in diffusion through the mucus is a mechanical obstructive effect as indicated by molecular weight, but additionally the flux is modified by chemical interactions between the diffusant and the constituent molecules of the mucus gel. The positively charged β-blocker molecules were retarded by a significantly greater amount than the negatively charged carboxylic acid.

It is however difficult to draw a general structure-activity relationship to predict which molecules may traverse the mucus gel more readily and which may have limited absorption due to glycoprotein-drug interactions in view of the results obtained for NP. Where the amide, NP, with a blocked positive charge was retarded by a significantly greater amount than the amino acid TRP possessing a free positively charged amino group.

The barrier that mucus presents to the transport of the small molecules tested in this study is not excessively large and all the compounds were able to penetrate the gel. This, however may not be true for larger peptidic molecules and needs to be clarified.

CHAPTER 5

THE EFFECT OF ABSORPTION ENHANCERS UPON DIFFUSION THROUGH MUCUS

CHAPTER 5

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5.1 INTRODUCTION

The nasal mucosa has been shown to be a site from which systemic absorption of a number of molecules with a molecular weight of up to 1000 can occur (McMartin et al., 1987). However, the absorption of larger molecular weight species is poor, which has prompted interest in identifying molecules and methods which can safely enhance absorption by this route. The methods pursued to date include improved formulations and delivery systems for nasal administration and the coadministration of additional compounds, termed absorption enhancers, to promote transmucosal absorption.

The aim of the formulation approach has been to extend the contact time of the drug within the nasal cavity and hence produce an increase in absorption. The contact time is dependent upon the clearance of the drug from the nasal cavity by the mucociliary clearance mechanism. This line of research has been endorsed by a recent theoretical model for nasal delivery, which describes the different rate processes involved (Gonda and Gipps, 1990). The model predicts that reducing mucociliary clearance may improve systemic absorption and additionally reduce variability in nasal drug absorption. As stated in chapter 1, the normal half-life of clearance is about 20 min (Aoki and Crawley, 1976; Gizururarson, 1990) although considerable intra- and inter-subject variation is observed.

Illum et al. (1987) demonstrated that albumin, starch and diethylaminoethyl (DEAE) - Sephadex microspheres had remarkably increased half-time clearance values of 3 hr, or more. Subsequently, degradable starch microspheres (DSM) have been evaluated for the nasal delivery of the poorly absorbed antibiotic gentamicin (Illum et al., 1988), insulin (Farraj et al., 1990), biosynthetic human growth hormone (Illum et al., 1990) and the decapeptide DDAVP (Critchley, 1989) to sheep. Significant increases in bioavailability (3-4 fold for DDAVP) were obtained for each compound, although the values were

all below the i.v. values. Recent work performed with DSM and CACO-2 cells has suggested that the mechanism of enhancement is by inducing an increased permeability of the tight junctions (Bjork et al., 1991), which may be additional to the effect upon mucociliary clearance.

The success of DSM has prompted the evaluation of other mucoadhesive delivery systems to increase nasal bioavailability. Mucoadhesives are compounds which can adhere to a biological surface for an extended period of time, as a result of an interaction with the surface mucus layer (Critchley, 1989). Mucoadhesive gels have been prepared using polyacrylic acid aqueous gel (Carbopol gel) and shown to significantly increase the absorption of insulin and eel calcitonin via the nasal cavity in rats (Morimoto et al., 1985). The bioavailabilities of vasopressin and its analogue 1-deamino-8-arginine vasopressin after nasal administration were increased by more than 2- and 1.6-fold respectively after administration in sodium hyaluronate solutions, which are both viscous and mucoadhesive (Morimoto et al., 1991). Critchley (1989) demonstrated that nasal administration of DDAVP to rats could be enhanced by carbopol (2% w/v), an anionic polymer of polyacrylic acid, and by an aqueous solution of the cationic polymer DEAE-Dextran (100 mg mL^{-1}) to bioavailabilities of 78% and 59% respectively. The use of DEAE-Sephadex spheres is also under evaluation for nasal delivery of insulin (Ryden and Edman, 1991).

Work has also been carried out to develop mucoadhesive microspheres (produced by cross-linking polyacrylic acid with maltose in a W/O emulsification process), for the nasal delivery of oxytocin and this is currently being evaluated in rabbits and humans (Lewis and Kellaway, 1990 a and b). The fact that polyacrylic acid does indeed impede the clearance of microspheres, has been demonstrated by Vidgren et al. (1991) using disodium cromoglycate and a double-labelling technique permitting the delivery of both the drug, and drug loaded microspheres simultaneously and hence overcoming intra-individual variation.

Other formulation approaches have evaluated the use of viscosity enhancers such as propylene glycol (Hardy et al., 1985) methylcellulose and hydroxypropyl methylcellulose (Harris et al., 1988; Pennington et al., 1988; Critchley, 1989). From these studies it is apparent that duration in the nasal cavity can be enhanced by increased solution viscosity. Critchley (1989) has also investigated a sustained release formulation for DDAVP using pluronic F-127 (27% w/v) in rats. The results demonstrated sustained absorption, but the overall extent of absorption was not significantly different to that obtained from solutions.

The second line of attack, which is the subject of this chapter, is the use of absorption enhancers. These are a large group of compounds with diverse chemical nature and mechanism of action. It is of note that few, if any, of these substances were originally designed for enhancing absorption. Hence, in most cases, the mechanism of enhancement is not fully understood and toxicological evaluation is essential.

It is difficult to categorise absorption enhancers but Lee (1990) has proposed a classification system of five major types. These classifications are: chelators; surfactants; bile salts and bile salt derivatives; fatty acids, bile salt-mixed micelles and phospholipids; non-surfactants such as unsaturated cyclic ureas. Some absorption enhancers do not however fall within these bands, but may usefully be classified as enzyme inhibitors, such as α-aminoboronic acid derivatives (Hussain et al., 1989), cyclopeptides such as bacitracin (Raehs et al., 1988; Sandow et al., 1990) and most recently cyclodextrins (Schipper, 1990 and 1991; Watanabe, 1991).

The potential biological barriers to nasal delivery have been reviewed in chapter 1 and comprise the mucus and periciliary layer, mucociliary clearance, enzymic degradation (luminally and intracellularly) and transport across the nasal epithelium. The mechanisms by which enhancers promote absorption is believed to be a consequence of

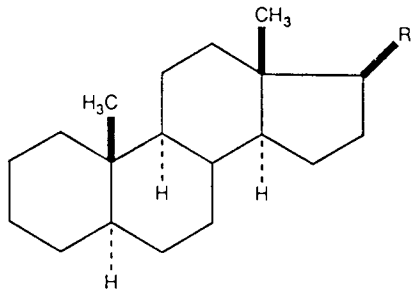
modification of one, or more of these barriers, or by altering the thermodynamic activity of the drug (Touitou et al., 1987).

A few absorption enhancers which are believed to act upon mucus will be discussed below. Bile salts, which have been used to promote nasal absorption by a number of workers (Hirai et al., 1981b; Gordon et al., 1985; Duchateau et al., 1986b and 1987), are reported to partly exert their affect by decreasing the viscosity of mucus. The evidence is that *in vitro* sodium deoxycholate, sodium glycholate and sodium taurodeoxycholate all reduced the viscosity and elasticity of bronchial mucus in a concentration-dependent manner, with 20 mM concentrations causing complete liquefaction of the mucus gel (Martin et al., 1978). The bile salt derivative sodium taurodeoxycholate (STDC) at 20 mM concentration similarly reduced the viscosity and elasticity of both native and purified pig gastric mucus (El-Hariri et al., 1989). Poelma et al. (1990b) have also reported that *in vitro* the bile salt taurocholic acid reduced the viscosity of pig intestinal mucus in a concentration-dependent manner up to the critical micelle concentration (8 mM), above this concentration the viscosity was not reduced further. The chemical structures of bile salts used in this study are illustrated in figure 5.1.

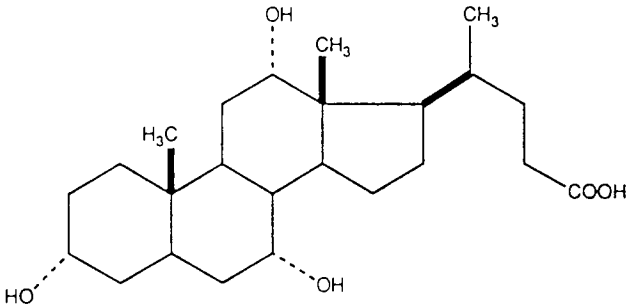
In addition to bile salts, the mucolytic agent N-acetyl-L-cysteine has been used as an absorption enhancer *in vivo* at a concentration of 20% w/v (which is the concentration used clinically to facilitate mucus removal in bronchopulmonary disease) and was found to significantly increase the intranasal absorption of synthetic growth hormone (a tripeptide). The total bioavailability was increased from 7.4% to 12.2 % (O'Hagan et al., 1990). This compound has been shown to reduce the viscosity and elasticity of purified gastric mucus at a concentration of 0.6M after 1 h incubation at 37°C (Walters, 1986). This effect is believed to be due to the reactive sulphydryl group breaking down disulphide bonds in the glycoprotein (Sheffner, 1963).

Figure 5.1 A diagram to illustrate the chemical structures of the different bile salts

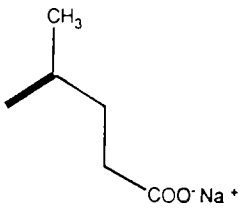
general steroid structure



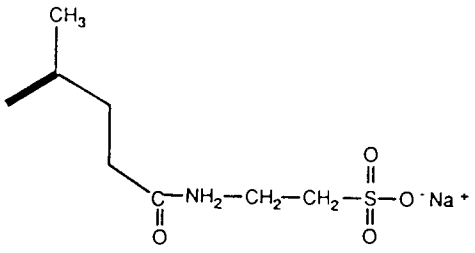
cholic acid



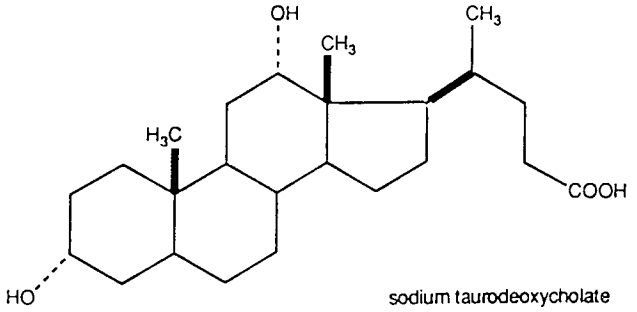
sodium cholate



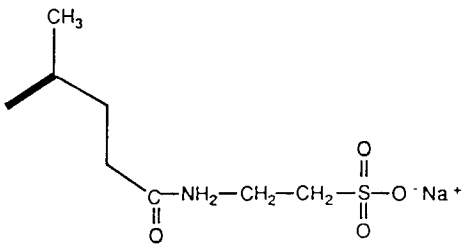
sodium taurocholate



deoxycholic acid



sodium taurodeoxycholate



The chelating agent EDTA has been used to promote the nasal absorption of insulin in rats (Aungst and Rogers, 1988a) and has been found to increase the bioavailability of the decapeptide DDAVP to 59% (Critchley, 1989). Its ability to promote absorption is related to its ability to chelate calcium and magnesium ions. These ions are important for maintaining the structure and rigidity of cells, and in particular for the integrity of the tight junctions between cells (Cassidy and Tidball, 1967; Suzuka et al., 1987), ciliary activity (Batts et al., 1989) and the maintenance of mucus gels (Marriott et al., 1979). EDTA has also been shown to alter transmucosal water influx (Shiga et al., 1985). Chelation of these ions could therefore promote absorption by interfering with one, or several of these phenomena.

In this study, absorption enhancers which possess the ability to alter mucus structure were investigated. The aim was to determine whether this ability was a major mechanism of enhancement, and hence to give an insight into the relative importance of the mucus layer as a barrier to peptide delivery. The compounds evaluated include the bile salts taurocholic acid, taurodeoxycholic acid and cholic acid, the chelating agent EDTA and the mucolytic N-acetyl-L-cysteine (NAC). The effect was investigated by monitoring the diffusion of a model compound, TRP, through mucus *in vitro*, so that any effect observed would be due solely to an effect upon mucus. Three-compartment diffusion cells as described in section 4.3.1 were employed.

5.2 THEORETICAL TREATMENT OF RESULTS

5.2.1 Calculation of flux rates

The flux values were determined as described in section 4.2.

5.2.2 Conversion of radioactive counts to molar concentrations

In each of the experiments a stock solution of TRP was prepared which had a concentration of 0.025M unlabelled TRP and contained 0.5 µL of [^{3}H]TRP per mL of unlabelled TRP. The radioactivity in the stock solution was determined by scintillation counting and was expressed as disintegrations per minutes (DPM).

The radiochemical purity of [^{3}H]TRP was 99% from the technical data. The specific activity of [^{3}H]TRP was 31.5 Ci/mmol from the technical data sheet. As 1 Ci is equivalent to 2.22×10^{12} dpm it follows that for 1 mole of [^{3}H]TRP there would be $(31.5 \times 10^3) \times (2.22 \times 10^{12})$ DPM, that is 6.993×10^{16} DPM.

$$\text{No of moles of } [^3\text{H}]\text{TRP} = \frac{\text{DPM in incubation mixture}}{6.993 \times 10^{16}} \qquad (5.1)$$

The molar concentration of [^{3}H]TRP was then determined by multiplying by 1000 and dividing by the sample volume. The total molar concentration of TRP and [^{3}H]TRP combined was then determined. The molar ratio of TRP : [^{3}H]TRP was determined to give a multiplication factor R. This procedure was determined for each stock solution used experimentally.

$$R = \frac{\text{concentration of unlabelled TRP (0.025M)}}{\text{concentration of } [^3\text{H}]\text{TRP in stock solution}} \qquad (5.2)$$

The protocol for the samples was as follows. The radioactivity expressed as DPM was converted to number of moles by equation 5.1, which was then expressed as a molar concentration. The molar concentration for [^{3}H]TRP was multiplied by R, determined from equation 5.2, to give the total concentration of TRP and [^{3}H]TRP for that sample.

5.3 EXPERIMENTAL

5.3.1 GENERAL PROCEDURE

In these experiments three-compartment diffusion cells were employed and assembled as described and illustrated in section 4.3.1. All experiments were performed at 20°C. Experimental protocol was as described in section 4.3.3.1.

5.3.2 Effect of 20 mM taurocholic acid (added at 240 min) on the diffusion of 0.025M tryptophan through mucus.

The experimental procedure described in section 5.3.1 was followed. Half-way through the experiment (at 240 min) the sodium salt of taurocholic acid (TA) was carefully added to the donor compartment as a powder to avoid altering the concentration of TRP in the donor compartment. Sufficient TA was added to produce a 20 mM concentration of the compound in the donor compartment. The experiment was allowed to proceed for a further 240 min.

Samples (100 µL) of each time sample were transferred to a separate test tube to which 100 µL of I.S. solution (phenylalanine 5 mM) were added and the mixture made upto 1 mL with phosphate buffer (pH 7.4). Analysis was performed with HPLC as described in section 2.3.1.1

5.3.3 Effect of 20 and 40 mM taurodeoxycholic acid (added at 240 min) on the diffusion of 0.025M tryptophan through mucus.

The experimental procedure described in section 5.3.1 was followed. At 240 min the sodium salt of taurodeoxycholic acid (TDA) was carefully added to the donor compartment as a powder. Sufficient TDA was added to produce a 20 mM concentration of the compound in the donor compartment of two diffusion cells and 40 mM in two other cells. The experiments were allowed to proceed for a further 240 min.

Samples (100 µL) of each time sample were transferred to a separate test tube to which 100 µL of I.S. solution (phenylalanine 5 mM) were added and the mixture made upto 1 mL with phosphate buffer (pH 7.4). Analysis was performed with HPLC as described in section 2.3.1.1

5.3.4 To determine the integrity of the tritium-tryptophan bond

The integrity of the tritium-TRP bond was determined to ensure that the radioactivity counted in the receiver compartment after diffusing through the TRP layer was due to TRP not simply the radioactive label. This was determined by reversed-phase HPLC connected to a fraction collector.

The HPLC system employed comprised a Cecil CE1100 liquid chromatography pump, a Rheodyne 7125 injection port fitted with a 20 µL sample loop and a Cecil HPLC CE1220 variable wavelength detector. Chromatography was performed on a 10 x 4.6 cm (length x internal diameter) column packed with 5 µm ODS-Hypersil reversed-phase material. The analytical column was protected by 1 x 4.6 cm guard column packed with the same 5 µm ODS-Hypersil reversed-phase material.

The system was equilibrated with a mobile phase comprised of 3 % acetonitrile and 0.1 % diethylamine in water, which was adjusted to a pH of 3.5 with orthophosphoric acid. This was pumped at a flow rate of 1.5 mL min^{-1}. The chromatograms were detected at 280 nm and recorded on an Omniscribe D500 chart recorder.

A 1 mM solution of TRP was injected onto the column to determine the retention time. A solution of 0.025M TRP containing 0.5 µL of [^{3}H]TRP per mL of unlabelled TRP, prepared for use in the diffusion experiments to be described below was then injected on to the column. Specific details of the radiolabelled compound are given in appendix 3. The column eluate was immediately connected to a fraction collector to collect and separate the mobile phase and test solution into suitable time-dependent samples (30 seconds).

The fraction collector employed was a programmable Gilson FC 203 fraction collector. The instrument was programmed to collect samples for thirty second intervals commencing at time = 0, when the TRP solution was injected onto the column, up to 10 min. This produced twenty samples in total.

A sample (0.5 mL) of eluate from each time sample was pipetted into a scintillation vial. Optiphase Hisafe 3 scintillation cocktail (10 mL) was dispensed into each vial and the vial contents were mixed by vortexing. The radioactivity in each sample was counted by a 2000CA TRI-CARB Liquid Scintillation Analyser produced by Packard United Technologies. The samples were counted using protocol 12, a tritium counting program. This protocol counted the number of

disintegrations occurring within five minutes for each individual sample.

5.3.5 Effect of 50 mM concentrations of penetration enhancers added at 120 min on the diffusion of 0.025M tryptophan through mucus.

The procedure was essentially as that described in section 5.3.1 with a few alterations.

The TRP solution used in these experiments contained 0.025M of "cold" unlabelled TRP with an additional 0.5 µL of [^{3}H]TRP per mL of unlabelled solution. The additional [^{3}H]TRP did not cause a significant increase in the total molar concentration of TRP in the donor solution.

At 120 min (rather than 240 min) the sodium salt of cholic acid (CA) was carefully added to the donor compartment as a powder. Sufficient CA was added to produce a 50 mM concentration of the compound in the donor compartment. This was repeated in triplicate. The experiments were allowed to proceed for a further 360 min. samples (0.4 mL) of each time point sample (0.5 mL) were pipetted into a scintillation vial. Optiphase Hisafe 3 scintillation cocktail (10 mL) was dispensed into each vial and the vial contents were mixed.

This procedure was followed to investigate the effect of 50 mM EDTA, 50 mM N-acetyl-L-cysteine (NAC). The protocol was also used to investigate the addition of 20 mM TA at 120 min in comparison with the addition of 20 mM TA at 240 min, on the diffusion of TRP through mucus.

The concentration of TRP in each case was determined by scintillation counting as described in section 5.3.4.

5.3.6 Effect of direct mixing of taurocholic acid with mucus on the diffusion of tryptophan through mucus.

Equal masses of TA were added to individual portions of pig gastric mucus (each 0.6g) and the bile salt incorporated into the gel by

gentle stirring with a glass rod. The masses of TA were 0.120g and 0.3098g, such that they would produce a 20 or 50 mM concentration of the compound in the donor compartment respectively(i.e. the same concentrations as used in section 5.3.5). The experiments for both concentrations was performed in triplicate. The mass of mucus chosen was that found empirically to fill the 1 mm thick space in the central compartment.

The cells were then assembled with the mixed mucus and bile salt within the central compartment and the procedure outlined in section 5.3.1 followed.

5.3.7 Control experiments

Control experiments were performed utilising an aqueous unstirred layer in place of the mucus layer to determine whether the observed changes in diffusion rate were due to enhancer-mucus or enhancer-TRP interactions.

A sample (0.31 mL) of phosphate buffer (pH 7.4) was added to the receiver compartment, which diffused into and filled the central compartment and created the aqueous unstirred layer. The procedure thereafter was the same as described in section 5.3.1. These experiments will be referred to hereafter as control.

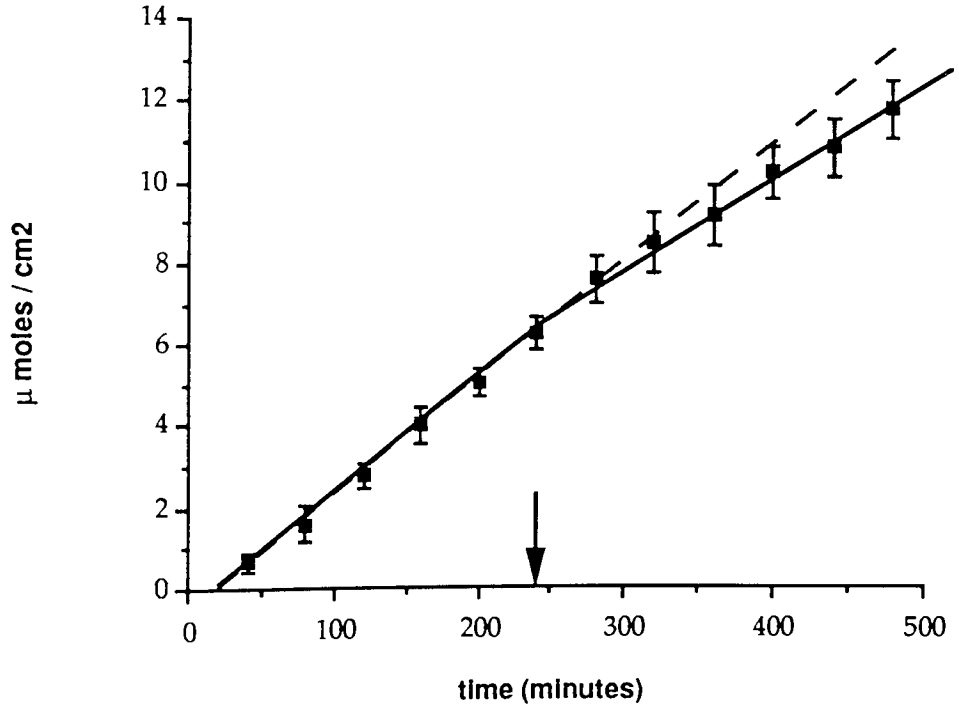
5.3.8 To investigate the complex-formation between tryptophan and taurocholic acid by UV-spectrophotometry.

A Phillips PU8730 UV/VIS spectophotometer was used to scan the solutions in the range of 200-600 nm in absorbance mode. Scans of TRP in phosphate buffer (pH 7.4) were performed with phosphate buffer as the blank. TRP concentrations were 2 and 0.5 mM. Scans were also carried out of taurocholic acid (TA) in phosphate buffer (pH 7.4) using buffer as the blank in the concentration range of 10-100 mM. Scans were then performed of solutions containing both TRP (0.5 mM) and TA at concentrations of 50 and 75 mM with phosphate buffer as blank. Finally, a scan of 0.5 mM TRP and 75 mM TA was performed with 0.5 mM TRP as the blank reference solution.

5.4 RESULTS AND DISCUSSION

The effect of the addition of 20 mM TA upon the diffusion of 0.025M TRP through mucus was initially investigated by adding the bile salt (as a powder) to the donor solution half-way through the experiment (at 240 min) and then allowing the experiment to proceed for a further four hours. The reason for this was to use the initial part of the experiment, up to the addition of the enhancer, as a control. The analysis was by reversed-phase HPLC. The results obtained are illustrated in figure 5.2 and reveal a biphasic plot.

Figure 5.2 The effect of the addition of 20 mM taurocholic acid at 240 min on the diffusion of 0.025M tryptophan through a mucus layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of three determinations.



The initial linear portion comprises seven points and represents the diffusion of TRP in the absence of enhancer. The flux over this period was (284 ± 8.23) x 10-4 μ moles cm-2 min-1 with a linear correlation coefficient of 0.999. During the second part of the experiment, using a line through the last 7 points, (including 240), a lower flux of (224 ± 8.88) x 10-4 μ moles cm-2 min-1 was observed, with a correlation coefficient of 0.998.

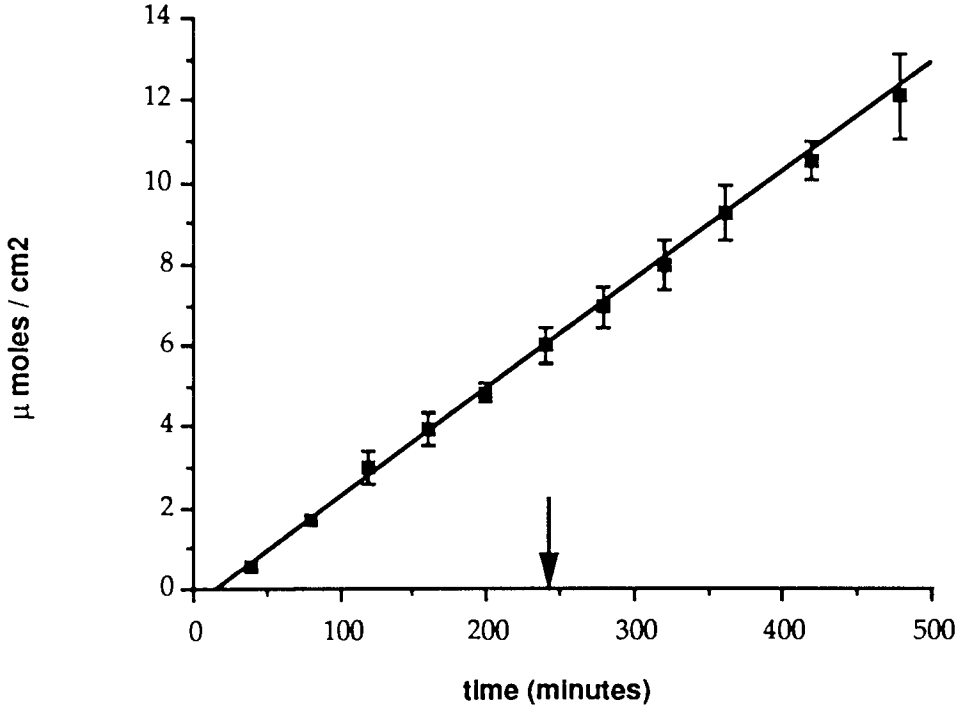
The calculated flux values suggested that TA may slightly reduce, rather than increase the rate of diffusion of TRP through mucus. This result was unexpected in view of research that has demonstrated that mixing mucus with 20 mM concentrations of bile salts *in vitro* significantly reduced the viscosity of both bronchial and pig gastric mucus (Martin et al., 1978; El-Hariri et al., 1989). This would presumably have increased the rate of diffusion by reducing the effectiveness of the diffusional barrier. Work performed by Turner et al., (1985) has demonstrated that pretreatment of native pig gastric mucus with 20 mM sodium taurodeoxycholate increased the rate of diffusion 1.5-fold, although the lag time was not affected.

Two possible explanations for not observing a marked alteration in the rate of diffusion of TRP upon the addition TA to the donor compartment may be proposed. The first is that insufficient bile salt was added to the donor compartment to reduce the viscosity of the mucus, or secondly that 4 h was insufficient time for the bile salt to dissolve, penetrate the mucus gel and exert any effect upon it.

To investigate the effect of increased concentration, the same protocol was employed to investigate the effect of 20 and 40 mM TDA on the diffusion of TRP through mucus. Linear plots were obtained for both 20 and 40 mM TDA and are illustrated in figure 5.3 a and b. The numerical calculated results are tabulated in tables 5.1 and 5.2.

Figure 5.3 The effect of the addition of taurodeoxycholic acid at 240 min on the diffusion of 0.025M tryptophan through a mucus layer of 1 mm thickness at a concentration of a) 20 mM and b) 40 mM. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of three determinations.

a)



b)

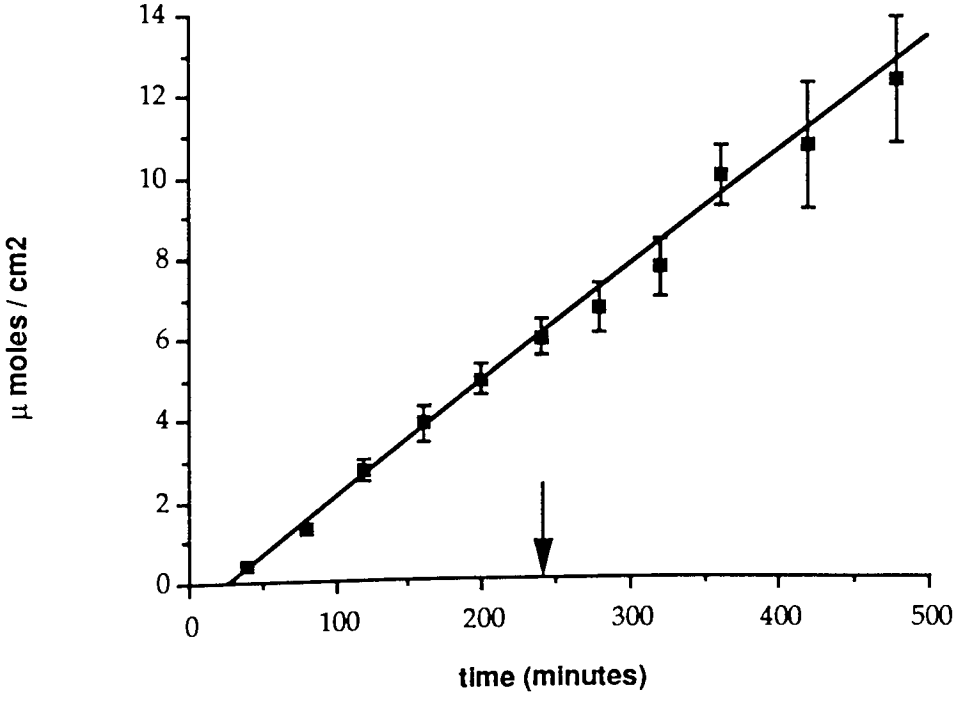


Table 5.1 Diffusion data for the effect of 20 mM taurodeoxycholic acid on the diffusion of 0.025M tryptophan through a mucus layer of 1 mm thickness. The data was calculated for: a) all twelve data points (WHOLE); b) first six data points up to and including 240 min (SECTION 1) and c) last six data points including 240 min (SECTION 2). Values are the mean ± sem of two determinations.

Data analysed	flux (x10⁴) μ moles cm⁻² min⁻¹	r
WHOLE	264 ± 18.5	0.999
SECTION 1	271 ± 20.3	0.998
SECTION 2	260 ± 19.9	0.999

There was no appreciable difference between the calculated diffusion rates for 20 and 40 mM TDA. Additionally, there were no marked differences between the fluxes calculated for the 2 different sections of the graph. The variation between the two sections was comparable to that seen when a similar analysis was performed on data for TRP diffusing through mucus, when no penetration enhancer was added. The results of this analysis are illustrated in table 5.3. This data did not demonstrate biphasic behaviour.

Table 5.2 Diffusion data for the effect of 40 mM taurodeoxycholic acid on the diffusion of 0.025M tryptophan through a mucus layer of 1 mm thickness. The data was calculated for: a) all twelve data points (WHOLE); b) first six data points up to and including 240 min (SECTION 1) and c) last six data points including 240 min (SECTION 2). Values are the mean ± sem of two determinations.

Data analysed	flux (x10⁴) μ moles cm⁻² min⁻¹	r
WHOLE	268 ± 37.5	0.998
SECTION 1	285 ± 30.6	0.997
SECTION 2	276 ± 50.0	0.987

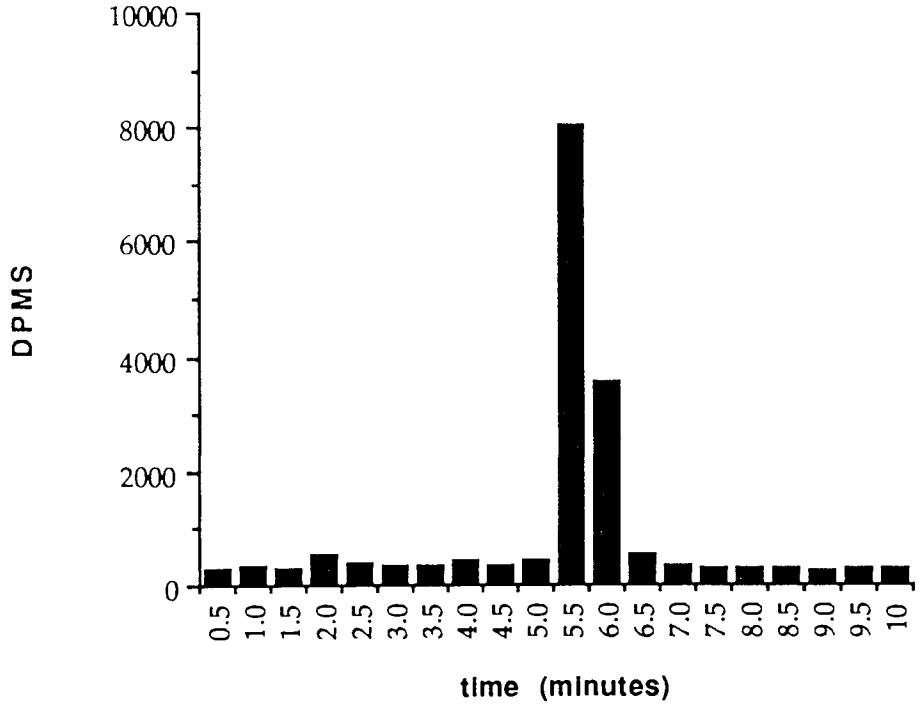
Table 5.3 Diffusion data for the diffusion of 0.025M tryptophan through a mucus layer of 1 mm thickness without the addition of an absorption enhancer. The data was calculated for: a) all twelve data points (WHOLE); b) first six data points up to and including 240 min (SECTION 1) and c) last six data points including 240 min (SECTION 2). Values are the mean ± sem of two determinations.

Data analysed	flux ($x10^4$) μ moles cm^{-2} min^{-1}	r
WHOLE	314 ± 5.44	0.999
SECTION 1	336 ± 11.37	0.999
SECTION 2	298 ± 1.20	0.997

This suggests that under these experimental conditions the bile salt TDA did not exert any effect upon the mucus.

In the experiments described so far HPLC was the analytical method. It was decided to use radiolabelled TRP and scintillation counting as the analytical method for the remainder of these studies, to enable the more rapid accumulation of data. The first step for these experiments was to determine the integrity of the tritium-TRP bond. The technical data pertaining to this compound, including its chemical structure, is presented in appendix 3. This was investigated by injecting a solution of 0.025M unlabelled TRP containing 0.5 μL of [^{3}H]TRP per mL of unlabelled TRP onto a reversed-phase HPLC column and collecting 20 samples of the mobile phase from 0 to 10 min with a fraction collector. The radioactivity in the individual fractions was determined by scintillation counting and the results are illustrated in figure 5.4

Figure 5.4 A diagram to illustrate the distribution of radioactivity in 30 second aliquots from 0 - 10 min of 0.025M unlabelled tryptophan containing 0.5 µL of [^{3}H]tryptophan mL^{-1} of unlabelled tryptophan, determined by reversed-phase HPLC and fraction collection.



One peak was observed on the HPLC chromatogram for the injection of unlabelled TRP and the solution containing unlabelled and radiolabelled TRP. The retention time of this peak was determined to be 6.4 min in both cases. The results in figure 5.4 indicate that there were two fractions which had higher radioactive counts than the background radiation. These occurred at time fractions 5.5 - 6.0 min and 6.0 - 6.5 min, which clearly corresponded to TRP. It was therefore concluded that the tritium label was attached to the TRP molecule and not to degradation products.

It was decided to investigate the effect of increasing the concentration of enhancer used to 50 mM. Additionally, the time for the enhancer to interact with the mucus gel was increased by adding the compound to the donor compartment at 120 rather than 240 min and allowing the diffusion experiment to proceed for a further 360 min. The effect of the enhancers EDTA, NAC and CA were investigated with this protocol.

The results obtained for the addition of 50 mM EDTA to the donor compartment at 120 min are illustrated in figure 5.5 and tabulated in table 5.4. A linear relationship was observed throughout and the flux, determined from all fifteen data points, was (280 ± 5.50) x 10^{-4} μ moles cm^{-2} min^{-1}. The correlation coefficient was calculated to be 0.999. Therefore it was concluded that EDTA did not alter the rate of diffusion of TRP through mucus under these experimental conditions.

The use of EDTA to promote nasal absorption has been reported for DDAVP (Critchley, 1989) and insulin (Aungst and Rogers, 1988) where bioavailabilities of 59 and 3.5 % respectively were obtained. The concentrations of EDTA used were 1% w/v for DDAVP and 5% w/v for insulin. In this study a concentration of 50 mM EDTA was used which corresponded to a 1.86% w/v solution. The concentration was therefore within the range where a promoting effect *in vivo* was observed although the mucus layer is this study is considerably thicker than that *in vivo*.

Figure 5.5 The effect of the addition of 50 mM EDTA at 120 min on the diffusion of 0.025M tryptophan through a mucus layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of three determinations.

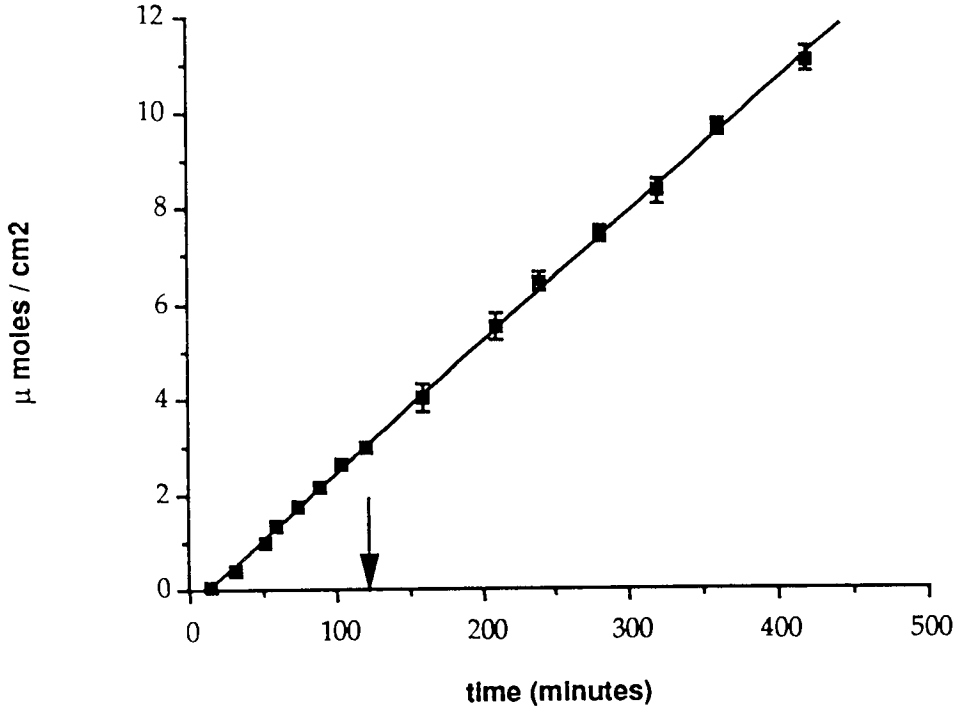


Table 5.4 Diffusion data for the effect of 50 mM EDTA on the diffusion of 0.025M tryptophan through a mucus layer of 1 mm thickness. The data was calculated for: a) all fifteen data points (WHOLE); b) first eight data points up to and including 120 min (SECTION 1) and c) last eight data points including 120 min (SECTION 2). Values are the mean ± sem of three determinations.

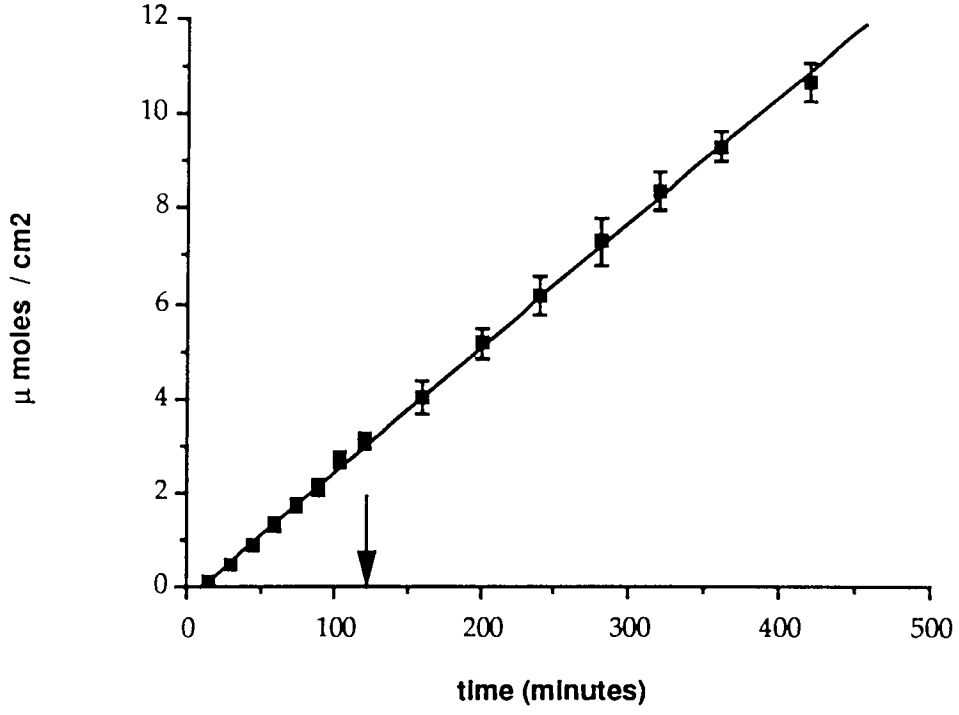
Data analysed	flux (x10⁴) µ moles cm⁻² min⁻¹	r
WHOLE	280 ± 5.50	0.999
SECTION 1	286 ± 3.22	0.999
SECTION 2	278 ± 6.76	0.999

Another possibility for the lack of an observed effect on mucus is that EDTA had difficulty in penetrating the mucus layer. Cherma et al. (1988) have reported that EDTA binds avidly to purified bronchial mucus and that it took three times as long for EDTA to diffuse out of a capillary layer of mucus than tritiated water. Calcium ions are essential for maintaining the mucus gel (Marriott et al., 1979) and additionally ionic abnormalities have been implicated in production of excessively thick and sticky mucus characteristic of cystic fibrosis (Phillips et al., 1990). EDTA however, is a non-selective chelating agent, unlike cyptands and crown ethers and therefore it possesses the ability to chelate other ions in addition to calcium (Touitou, 1991). It is perceivable that the EDTA has chelated insufficient calcium ions in the mucus, due to competition with other ions, for any significant difference in the mucus gel to have been created and hence no difference in the diffusion rate *in vitro* was observed. Lieberman (1968) reported that concentrations in excess of 0.25M were required for EDTA to exert a mucolytic effect, whilst a concentration range of 1-2.7 x 10^{-3}M was ineffective.

The alternative conclusion from this result would be that EDTA does not exert its promoting effect by interacting with the mucus gel, which would be in agreement with List et al. (1978) who observed no effect of 5 mM EDTA on pig gastric mucus. This would be consistent with the fact that EDTA exerts an enhancing effect by other mechanisms which are excluded from this model. It has been suggested that EDTA affects the tight junctions interconnecting membrane cells and consequently increases paracellular and pore transport (Cassidy and Tidball, 1967; Suzuka et al., 1987). Batts et al. (1989) have demonstrated with the frog palate *in vitro* that EDTA irreversibly halts mucociliary clearance, which, if such an effect occurred *in vivo*, would increase contact time with the nasal mucosa and may promote absorption.

The effect of the addition of 50 mM of the mucolytic NAC on the diffusion of TRP through mucus is illustrated in figure 5.6. A linear relationship was again observed and a line drawn through all fifteen

Figure 5.6 The effect of the addition of 50 mM N-acetylcysteine at 120 min on the diffusion of 0.025M tryptophan through a mucus layer of 1 mm thickness. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of three determinations.



points gave a flux of (268 ± 10.5) x 10^{-4} µ moles cm^{-2} min^{-1} and a correlation coefficient of 0.999. It was concluded that NAC had no effect upon the rate of diffusion of TRP through mucus under these experimental condition. The numerical calculated data for NAC is tabulated in table 5.5.

Table 5.5 Diffusion data for the effect of 50 mM N-acetylcysteine on the diffusion of 0.025M tryptophan through a mucus layer of 1 mm thickness. The data was calculated for: a) all fifteen data points (WHOLE); b) first eight data points up to and including 120 min (SECTION 1) and c) last eight data points including 120 min (SECTION 2). Values are the mean ± sem of three determinations.

Data analysed	flux (x 10^4) μ moles cm^{-2} min^{-1}	r
WHOLE	268 ± 10.5	0.999
SECTION 1	290 ± 16.2	0.998
SECTION 2	260 ± 5.70	0.999

This result was surprising as it was expected that the mucolytic agent would reduce the viscosity of the mucus gel and consequently that the rate of diffusion would increase. An increase in diffusion rate has been reported by Turner et al (1985). These workers investigated the effect of 100 mM NAC on the rate of diffusion of hydrogen ions through native gastric mucus using three-compartment diffusion cells. They observed that the rate of diffusion through the mucus pretreated with NAC was almost 1.5 times faster than through untreated mucus. A reduction in lag time from 9.88 to 2.94 min was also observed. The workers proposed that the increased rate of diffusion and reduced lag times were consistent with reducing disulphide bonds and lowering the viscosity of the gel. It should be noted however that the concentration of NAC used by Turner et al. was twice that used in this study. Additionally, it should be noted that the mucus was dialysed against 100 mM NAC overnight, which gave the mucolytic agent considerably longer to penetrate and interact with the mucus than in this study. It has been observed that NAC will only reduce the viscosity of mucus if the two are well mixed (Livingstone, 1991). It is possible that the mixing process may breakdown structure by shearing the mucus. Marriott et al. (1983) suggested from their work with NAC, that considerable time was required for the compound to

exert its effect and that the majority of disulphide groups were masked by entangled side chains of the macromolecule. If NAC were to be used as an intranasal absorption enhancer *in vivo*, such a pretreatment and mixing step would be impossible. The compound would also only have a short half-life within the nose due to clearance by the mucociliary clearance mechanism.

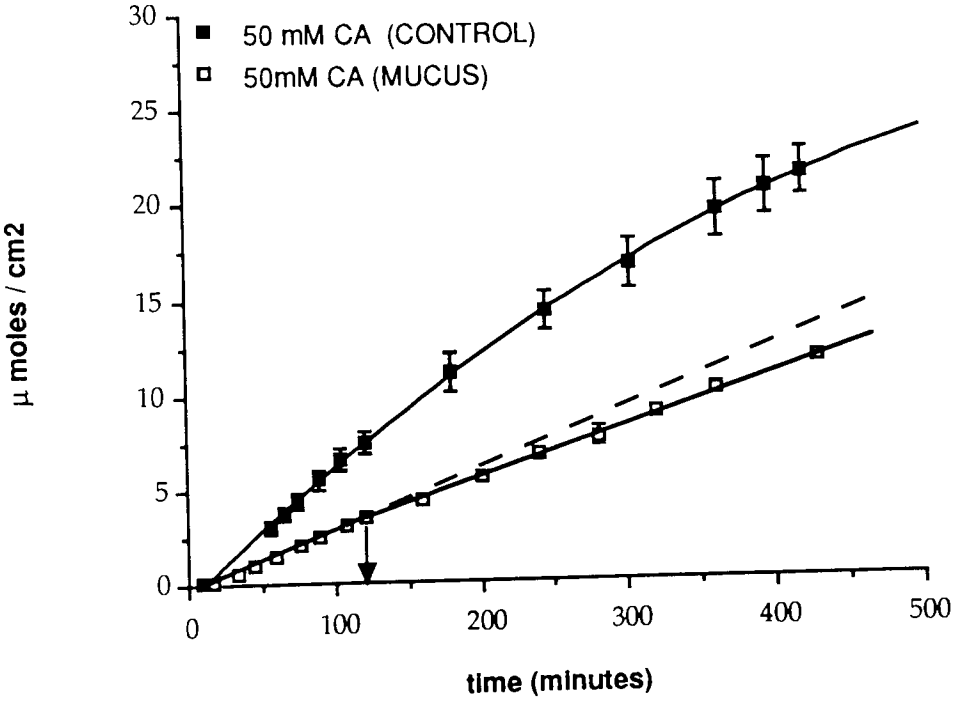
In contrast to the work of Turner at al. (1985) are the observations of Cheema (1985) who demonstrated that NAC (100, 200 and 300 mM concentrations) produced a concentration-dependent decrease in the permeability of native gastric mucus as illustrated by the diffusion coefficients for tritiated water and benzylpenicillin. Interestingly, no such effect was observed for diffusion through purified pig gastric mucus. Again, the concentrations of NAC used were considerably higher than those in this study and the mucus was dialysed overnight against the mucolytic agent. In the study by Cheema, no account was made for the change in pH induced by such high concentrations of NAC.

The different effects observed with crude and purified gastric mucus suggests that non-mucin components of the mucus gel may be involved. The preparation used in this study contained high concentrations of protein in addition to protein contained within the glycoprotein molecules. These molecules may contain disulphide bonds and hence be degraded by NAC in addition to, or in preference to the glycoprotein molecules. Another possibility is that the proteins may inactivate the drug, leading to a less dramatic decrease in gel structure (Marriott et al., 1983)

This reduction in permeability elicited by 5 % NAC has also been reported by Saggers and Lawson (1966), who investigated the diffusion of eighteen different antibiotics through artificial gastric mucin pretreated with NAC for 1 hr at 37°C.

The results for the addition of 50 mM CA on TRP diffusion through mucus is illustrated in figure 5.7 and reveals a biphasic plot.

Figure 5.7 The effect of the addition of 50 mM cholic acid at 120 min on the diffusion of 0.025M tryptophan through a mucus layer of 1 mm thickness (MUCUS) and through an aqueous unstirred layer of the same thickness (CONTROL). Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of three determinations.



The numerical results are tabulated in table 5.6. These results suggested that as in the case of TA, the enhancer CA did not increase the rate of flux but in may in fact reduce it. The difference between the two rates was not significant ($p < 0.05$).

Table 5.6 Diffusion data for the effect of 50 mM cholic acid on the diffusion of 0.025M tryptophan through a mucus layer of 1 mm thickness. The data was calculated for: a) all fifteen data points (WHOLE); b) first eight data points up to and including 120 min (SECTION 1) and c) last eight data points including 120 min (SECTION 2). Values are the mean ± sem of two determinations.

Data analysed	flux (x 10^4) μ moles cm^{-2} min^{-1}	r
WHOLE	282 ± 5.50	0.999
SECTION 1	325 ± 9.24	0.999
SECTION 2	270 ± 0.577	0.999

The control curve for 50 mM CA, where the enhancer was added to the donor phase at 120 min to investigate the effect of CA on the diffusion of TRP through an aqueous layer, is also illustrated in figure 5.7. As for the aqueous control experiments discussed in chapter 4, the rate profile is initially linear followed by curvature in the latter stages. The implications of this control curve will be discussed later in this section.

The addition of 20 mM TA at 120 min produced a biphasic plot, as observed when 20 mM was added at 240 min. This is illustrated in figure 5.8 and the tabulated results in table 5.7. The reduction in flux observed for the second stage of the plot was not appreciably different to that obtained if the enhancer was added at 240 min.

Figure 5.8 The effect of the addition of 20 mM taurocholic acid at 120 min on the diffusion of 0.025M tryptophan through a mucus layer of 1 mm thickness in comparison with the addition of 20 and 50 mM TA directly to mucus. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of three determinations.

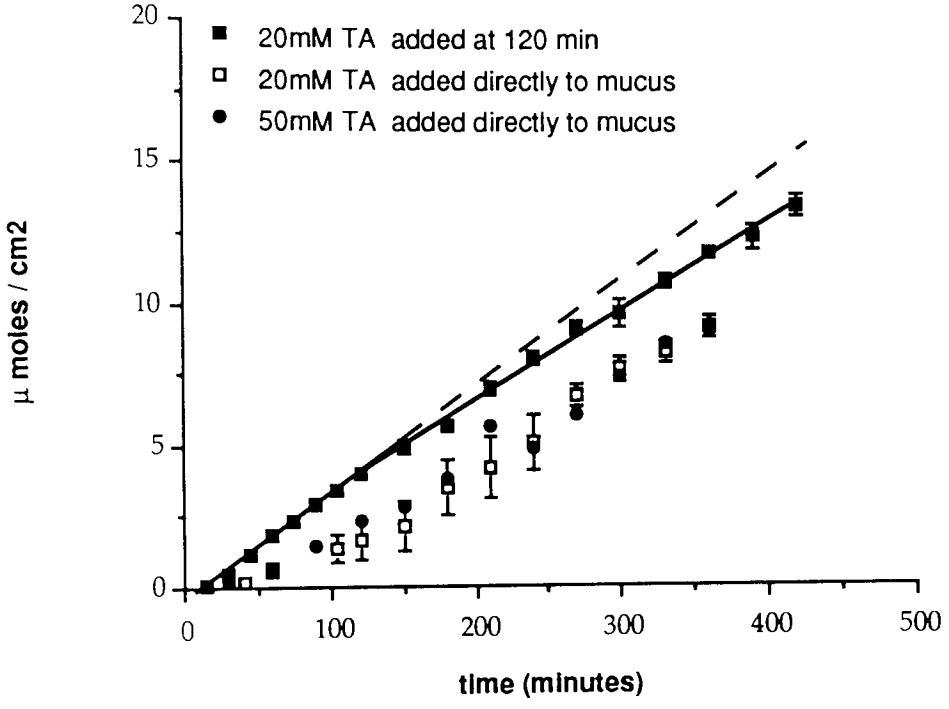


Table 5.7 Diffusion data for the effect of 20 mM taurocholic acid added at 120 min on the diffusion of 0.025M tryptophan through a mucus layer of 1 mm thickness. The data was calculated for: a) all fifteen data points (WHOLE); b) first eight data points up to and including 120 min (SECTION 1) and c) last eight data points including 120 min (SECTION 2). Values are the mean ± sem of two determinations.

Data analysed	flux (x 10^4) μ moles cm^{-2} min^{-1}	r
WHOLE	330 ± 11.0	0.999
SECTION 1	381 ± 22.5	0.998
SECTION 2	316 ± 9.00	0.998

The results for the direct mixing of 20 or 50 mM TA with the mucus, are also illustrated in figure 5.8 and the tabulated results are presented in table 5.8. The addition of 20 and 50 mM TA directly to the mucus prior to assembling the cell both increased the lag time (54 and 34 min respectively) and reduced the rate of diffusion. This result suggested that the reduction in flux observed when the bile salts were added at 120 min was genuine.

Table 5.8 Diffusion data for the effect of 20 and 50 mM taurocholic acid (TA) added directly to mucus on the diffusion of 0.025M tryptophan through a mucus layer of 1 mm thickness.

concentration of TA (mM)	flux (x 10^4) μ moles cm^{-2} min^{-1}	lag time min	r
20	295 ± 9.93	54.23 ± 18.06	0.991
50	276 ± 27.3	39.91 ± 12.05	0.990

Why would the addition of bile salts reduce the permeability of mucus? One way that bile salts could reduce the observed rate of diffusion is if complex-formation occurred, or TRP became associated with bile salt micelles. The effect of such interactions would be to reduce the concentration of free drug and the concentration gradient. This, in turn, would reduce the rate of diffusion (Barry, 1983). The absorption of two lipophilic compounds, griseofulvin and ketoconazole *in vivo* was reduced by concentrations of 10 and 20 mM TA. The reduction in the absorption rates was in agreement with the decreased fraction of free drug in solution due to micellisation by TA (Poelma et al., 1990a). In addition to reducing the free drug concentration the complexes formed may also have greater difficulty in diffusing through the mucus because of their increased size.

To investigate if this were the mechanism reducing the rate of diffusion control experiments were performed. In these experiments the mucus layer was replaced by an aqueous layer of the same thickness. The bile salts were added to the donor compartment at 120 min at a concentration of 50 mM. The results obtained are illustrated in figure 5.9 and for comparison the graph of TRP through an aqueous unstirred layer in the absence of enhancer is included.

The profile of each of the three graphs is initially linear followed by curvature in the latter stages. The fluxes were determined for each graph by linear regression on the first nine data points and are presented in table 5.9. The curves were not markedly different to each other, which suggested that complex-formation or micellisation was not occurring and that any reduction in the rate of diffusion was due to some interaction with the mucus.

This was further investigated by performing UV scans from 200-600 nm on solutions of 0.5 mM TRP and varying concentrations of TA. The results did not show a peak shift for TRP or an absorbance change different to the individual components (data not shown). These suggested that no complex-formation or micellisation was occurring between the two components

Figure 5.9 The effect of the addition of 50 mM taurocholic acid (TA) and cholic acid (CA) at 120 min on the diffusion of 0.025M tryptophan through a 1 mm thickness aqueous unstirred layer in comparison with the diffusion of TRP in the absence of an enhancer. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of three determinations.

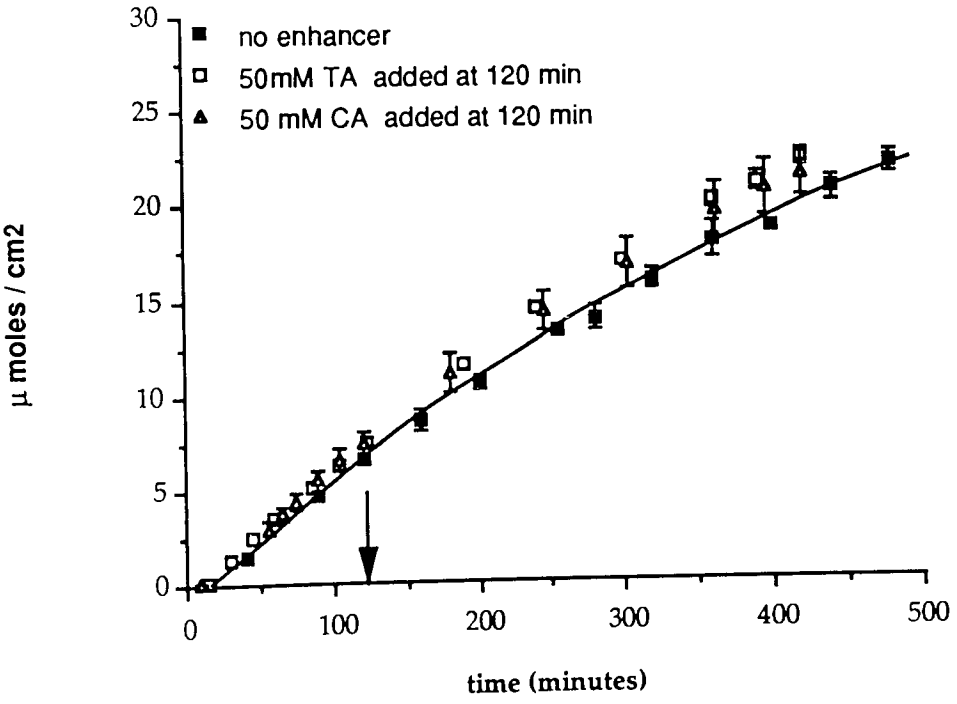


Table 5.9 Diffusion data for the effect of 50 mM taurocholic acid (TA) and cholic acid (CA) added at 120 min on the diffusion of 0.025M TRP through an aqueous unstirred layer of 1 mm thickness calculated from the first nine data points.

concentration of enhancer (mM)	flux (x 10^4) μ moles cm^{-2} min^{-1}	lag time min	r
0	520 ± 7.22	2.35 ± 0.97	0.998
50 TA	674 ± 24.0	9.88 ± 2.95	0.999
50 CA	612 ± 42.3	2.98 ± 2.50	0.997

The result of the addition of the bile salts TA and CA in these diffusion experiments suggest that bile salts rather than increasing the rate of diffusion through mucus, may reduce it. Evidence from diffusion through an aqueous a layer and UV spectroscopy suggests that this reduction is not due to micellisation and implies that bile salts do alter the rheological nature of mucus in contrast to the ideas of Bell et al. (1985) although rheological evaluation of the effect of the different enhancers on the mucus preparation used in this study was not evaluated. Cheema (1985) suggested that it was the microviscosity which was important in controlling the permeability of mucus rather than the macroviscosity, which would be measured as elasticity and viscosity changes *in vitro*. He proposed that the mucus gel could be considered as a lattice network with tortuous channels through which diffusing molecules must pass. Treatment with mucolytics partially destroyed the primary or secondary stabilisation of the gel and allowed adjacent chains to reorientate, or collapse and move closer together and possibly aggregate. The sum total effect would be the reduction in the size and number of open channels containing water for diffusants to traverse.

The indications of this work are that penetration enhancers which reduce the macroviscosity of mucus gels may reduce the rate of flux through mucus rather than increase it. This action appears to negate

their use as absorption enhancers by acting on mucus gels and may have major implications on the diffusion of larger peptidic molecules through mucus, where diffusion through the gel may be of greater significance and where the enhancing agent may compound the problem. This would be interesting to evaluate experimentally. Furthermore, the fact that bile salts, NAC and EDTA have been shown to promote nasal absorption *in vivo* suggests that mucus may not be the most important barrier to peptide delivery.

5.5 SUMMARY

The effect of the absorption promoters TA, TDA, CA, NAC and EDTA on the diffusion of 0.025M TRP through mucus was evaluated using three-compartment diffusion cells. The addition of the bile salts TA and CA to the donor compartment part way through the experiment produced a slight, but not significant reduction in the rate of diffusion of TRP. This result was confirmed by adding the bile salt TA directly to the mucus, where the lag times were increased and the rate of diffusion decreased. This effect was proposed not to be due to micellisation or complex-formation from the uniformity of diffusion of TRP through an aqueous unstirred control layer in the presence and absence of bile salts and from UV-spectrometry. This suggested that the effect was indeed due to interaction of the bile salt with the mucus gel.

The absorption promoters TDA, NAC and EDTA did not exert any effect on the rate of diffusion of TRP through mucus. This may be due to insufficient concentrations of the enhancing agents being used.

CHAPTER 6
BINDING OF DRUGS TO MUCUS

CHAPTER 6

BINDING OF DRUGS TO MUCUS

6.1 INTRODUCTION

Several studies have demonstrated binding effects between the glycoprotein molecules comprising the mucus gel and a variety of diverse compounds and organisms. One reason for this is the growing interest in using bioadhesion, specifically mucoadhesion, as a strategy for controlled drug delivery (Gu et al., 1988). Secondly, microbiologists and clinicians have been investigating the binding of pathogens, particularly the respiratory tract coloniser *Pseudomonas aeruginosa*, to mucus in order to gain a better understanding of pathogen establishment in a host (Vishwanath and Ramphal, 1985; Paerregaard et al., 1991). The other line of research is the interaction between drug molecules and mucus, which may impair drug absorption (Saggers and Lawson, 1966; Nimmerfall and Rosenthaler, 1980; Mayer et al., 1985; Kearney and Marriott, 1987; Hughes, 1988).

Many of the observed interactions between glycoprotein molecules and positively charged species have been attributed to electrostatic interactions as illustrated by the formation of complexes with positively charged monoquaternary ammonium compounds (Levine et al., 1955; Cavallito and O'Dell, 1958), various metal cations (Crowther and Marriott, 1984) and aminoglycosides (Ramphal et al., 1988). This type of interaction is likely in view of the chemical composition of the glycoprotein molecule. As described in chapter 1, two of the five sugars found in mucin may be sulphated and hence acquire a negative charge and additionally sialic acid is intrinsically negatively charged. The pKa values of sialic acid is 2.6 (Eylar et al., 1962), which results in the glycoprotein molecules being highly dissociated at most physiological pH values. Consequently the mucus gel behaves as a negatively-charged polyelectrolyte (Crowther and Marriott, 1984).

Electrostatic interaction is an important but not a sole mechanism of drug binding to mucus. Kearney and Marriott (1987) observed that significant binding of the antibiotic tetracycline to glycoprotein molecules occurred even if the drug was electrically neutral. They

proposed that the binding of this compound was due to simultaneous electrostatic and hydrophobic processes.

Hydrophobic interactions may at initial inspection appear unlikely in view of the observation that mucus is an extensively hydrated molecule. It has however been demonstrated that glycoprotein molecules contain a high molecular weight, hydrophobic protein as an integral part of their structure, as indicated by work on intestinal mucus from various animals (Pearson et al., 1981; Robertson et al., 1989) and human respiratory mucus (Naziruddin et al., 1990; Shankar et al., 1990). These hydrophobic regions may undergo hydrophobic interactions with the chemical species. A number of proteins, which could act in this manner have been shown to be associated with the mucus gel including serum albumin (Creeth, 1978), secretory globulins (Clamp, 1977), lysozyme and lactoferrin (Clamp and Creeth, 1984). In addition to protein groups, it has been suggested that associated and covalently-bound lipids in mucus contribute to the hydrophobic nature of mucins (Slomiany et al., 1988). The increased permeability of dog gastric mucus (Sarosiek et al., 1984) and reduced resistance to oxygen radicals (Gong et al., 1990) when these lipids were removed indicates that these molecules are important in maintaining the barrier properties of the mucus layer. This effect may be due to glycoprotein-lipid or lipid-drug interactions.

The binding of tetracycline to mucus has also been proposed to occur by chelation to cationic species (such as calcium) already associated with the mucin (Saggers and Lawson, 1966; Kearney and Marriott, 1987). Pre-incubation of mucin with the chelating agent EDTA reduced the binding of tetracycline, which would support this hypothesis (Saggers and Lawson, 1966). It has been suggested that the binding of this compound may also involve hydrogen bonding (Braybrooks et al.,1975)

The interaction of peptides and protein molecules with mucus may be a particularly important factor responsible for reduced drug absorption in view of the possibility of ionic and hydrophobic interactions with glycoprotein molecules or other proteins and lipids associated with

the gel. Albumin and mucin have been shown to interact strongly by non-covalent, hydrophobic binding producing a mucus-albumin complex which causes a significant increase in viscosity (List et al., 1978). This increase was greater than the sum of the two individual viscosities. Other proteinaceous biopolymers bovine serum albumin, IgG and IgA have also been shown to increase the viscosity of mucus by non-covalent hydrophobic, hydrophilic and ionic interactions (Marriott et al., 1981; Murty et al., 1984). The aim of these binding studies was to investigate the effect of different physicochemical properties of drug molecules on the degree of mucus-drug binding and any relationship between these results and the observed diffusivities described in chapter 4.

The methods used to investigate drug-mucus binding effects can be divided into the following classifications: equilibrium dialysis (Nimmerfall and Rosenthaler, 1980; Kearney and Marriott, 1987; Hughes, 1988); ultrafiltration (Saggers and Lawson, 1966; Cheema et al., 1988) and centrifugation (Saitoh et al., 1986). In this study the centrifugation method was employed. The basis of the technique is that the drug and mucus suspension are mixed freely, without the drug having to traverse a dialysis membrane, for a set time to allow binding to occur. The samples are then centrifuged at a high *g*-force (29,000*g*), which precipitates the mucus and drug-mucus complexes as a pellet. It is then possible to determine the concentration of free drug in the supernatant and hence determine the concentration of bound drug.

6.2 THEORETICAL TREATMENT OF BINDING RESULTS

The percentage of drug bound to the mucus, was determined from the concentration of drug bound to the mucus (D_b) and the total drug concentration (D_t) which was the concentration of the initial drug solution, according to the following equation:

$$\% \text{ Bound} = \frac{D_b}{D_t} \times 100 = \frac{D_t - D_f}{D_t} \times 100 \qquad (6.1)$$

where D_f is the concentration of free, unbound drug in the supernatant after incubation with the mucus suspension.

The interaction of a drug with a macromolecule can be described by the following equation:

$$\text{Drug} + \text{Macromolecule} \rightleftarrows \text{Drug/macromolecule complex} \qquad (6\cdot2)$$

The concentration of the bound drug may be related to the concentration of the unbound drug by the law of mass action:

$$K = \frac{r}{D_f(n-r)} \qquad (6.3)$$

Where K is the association (or affinity) constant, r is the concentration of bound drug D_b, divided by the concentration of macromolecule M, and n is the number of binding sites for the drug per macromolecule. In these experiments the concentration M was 1% w/v, which assuming a molecular weight of 2 x 10-6 corresponded to a molar concentration of 5 μM.

Equation 6.3 may be rearranged to give:

$$r = n\,\frac{K.D_f}{1 + K.D_f} \qquad (6.4)$$

It is usual to experimentally measure D_f and hence determine r, for a range of drug concentrations D_t. The values of n and K are then determined using a linear transformation of equation 6.4.

One of these transformations was proposed by Klotz et al. (1946). It is the reciprocal of equation 6.4 and produces the equation:

$$\frac{1}{r} = \frac{1}{nK} \cdot \frac{1}{D_f} + \frac{1}{n} \qquad (6.5)$$

Thus, a plot of 1/r versus $1/D_f$ should produce a linear plot with an intercept on the y-axis corresponding to 1/n and a slope of 1/nK as illustrated in figure 6.1a. A linear plot will only be obtained providing each molecule is bound to the same type of binding site (e.g. the same amino acid or group of amino acids on a protein), and binding of one molecule does not affect the binding of a second, or, third molecule in any way, except insofar as the first molecule reduces the number of spaces available to the second. If these conditions are not fulfilled curvature is produced in the plot. This may be due to the presence of more than one binding site for the compound under investigation, or inter-binding site interactions which render the same kind of binding sites non-equivalent.

A criticism of the Klotz transformation is that low values of free drug are given maximum weighting in linear regression analysis, while high ligand concentrations, where there is greatest analytical precision, have values that are clustered together near the origin and will be given low weighting.

Scatchard (1949) proposed an alternative reciprocal transformation. By rearranging equation 6.4 it can be shown that:

$$r + r\,K\,D_f = n\,K\,D_f \qquad (6.6)$$

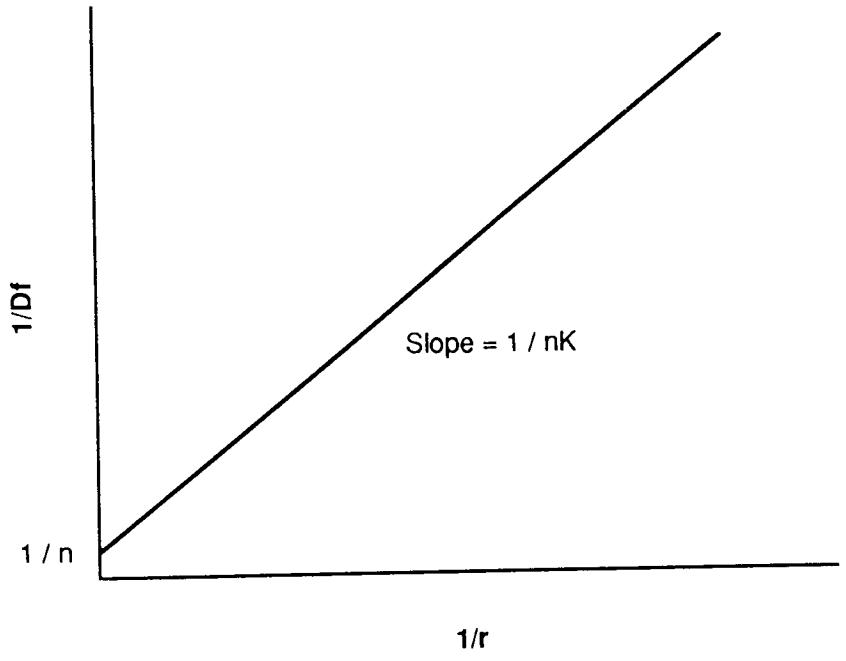
By dividing each side of the equation by D_f and rearrangement, it can be shown that:

$$\frac{r}{D_f} = nK - rK \qquad (6.7)$$

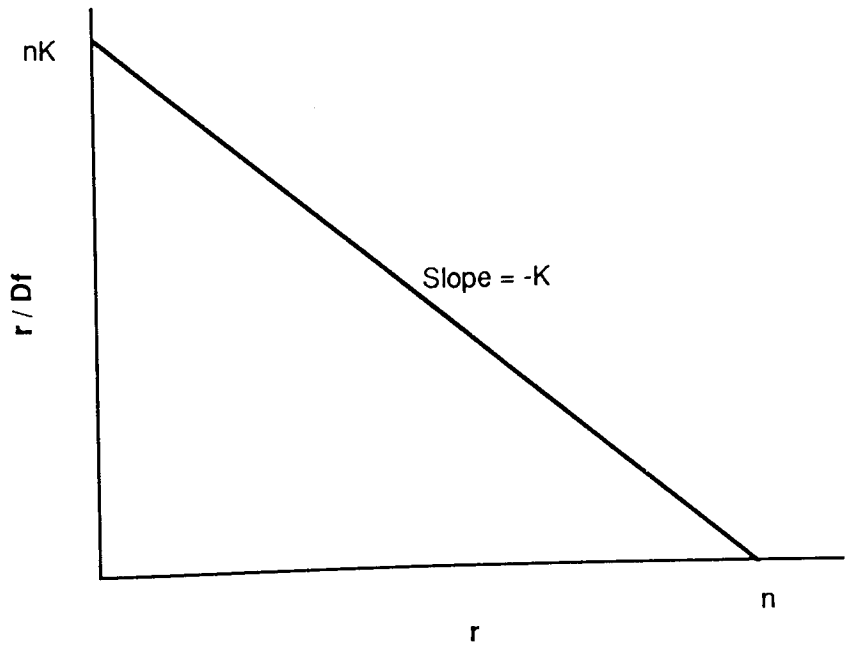
Therefore a plot of r/D_f against r should be linear with intercepts on the y-axis corresponding to nK and on the x-axis to n, as illustrated in figure 6.1b, providing, as for the Klotz transformation that there is a single class of non-interacting binding site. A deviation from linearity is again proposed to demonstrate the existence of more than one binding site, or interaction between the binding sites.

Figure 6.1 A schematic representation of: a) a Klotz plot and b) a Scatchard plot where there is one class of non-interacting binding site.

a)



b)



A third method of binding data analysis is the Freundlich adsorption isotherm. The equation is an empirically derived adsorption equation which describes the effect of pressure on the adsorption of gases onto a solid adsorbate:

$$V = a.p^{1/n} \qquad (6.8)$$

where V is the equilibrium volume of gas adsorbed per unit mass of adsorbent at a pressure, p and a and n are constants. The derivation of the equation assumes that the surface of the adsorbent is heterogeneous and as such that the heat of adsorption varies with the surface coverage (Shaw, 1980; Adamson, 1990)

Adsorption is a surface effect whereby a solid takes up a liquid or gas concentrated at its surface. The forces involved may be non-specific (van der Waal's) forces or stronger specific forces which are operative in the formation of chemical bonds. The Freundlich equation is frequently applied to adsorption from solution data by substitution of a concentration term for the pressure term in equation 6.8 which yields:

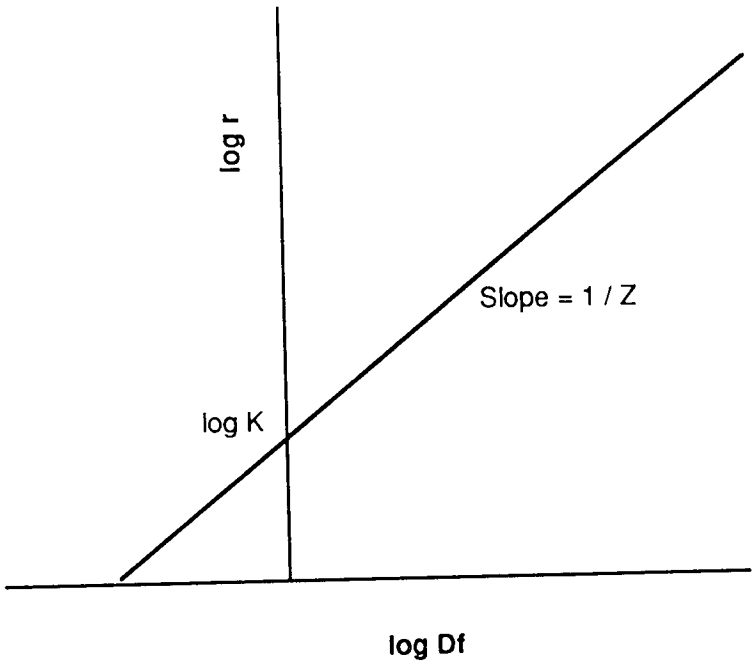
$$r = a.D_f^{1/n} \qquad (6.9)$$

or in its logarithmic form:

$$\log r = \log a + \frac{1}{n} \log D_f \qquad (6.10)$$

If the equation is obeyed, a plot of log r against log D_f should be linear with slope corresponding to 1/n and the ordinate intercept corresponding to log a and would indicate that adsorption occurred at non-equivalent sites. This is illustrated in figure 6.2. The constants a x n give a measure of the adsorbent capacity and the slope 1/n of the intensity of adsorption. The value of n is typically greater than 1 (Adamson, 1990). This type of analysis has been used by Hughes (1988) for the analysis of drug-mucin binding.

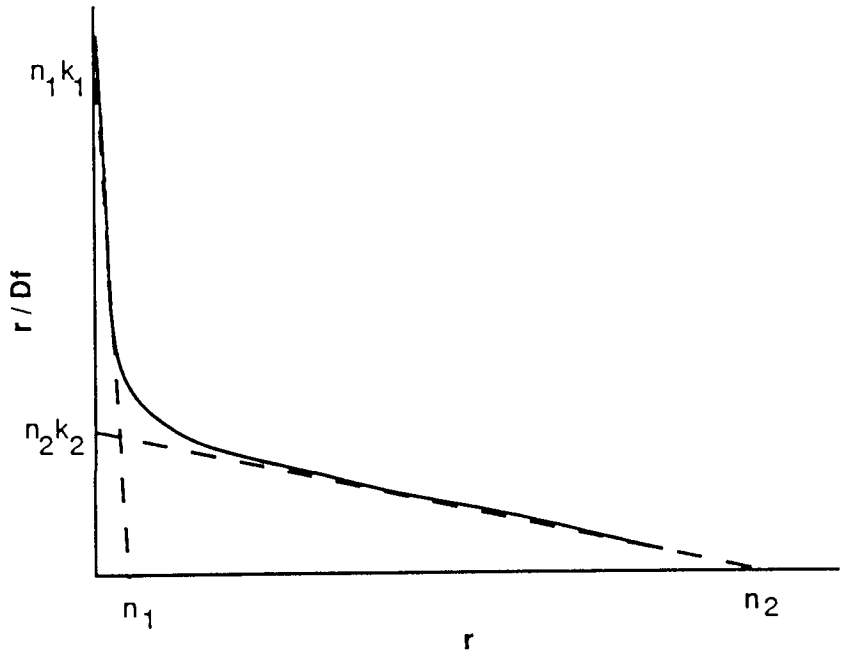
Figure 6.2 A schematic representation of a Freundlich plot.



While both the Klotz and the Scatchard methods have been widely used in analysis of binding data and a number of computer programs are available to treat the data, it is acknowledged that both the Scatchard and Klotz methodology are suitable only when one class of non-interacting binding site is present, when these conditions are not fulfilled the results at best are approximate (Vallner, 1977). Concave curves, as illustrated in figure 6.3, are frequently generated using the Scatchard method of analysis. This has been taken to infer that the protein provides two different classes of binding site. The one binding site is considered to have high affinity and low capacity and the other low affinity and high capacity. The methodology to determine K and n for the two classes of binding site under these circumstances has involved extrapolation of the two tangents of the curve to either asymptote on the graph. The intercepts on the y-axis and x-axis then correspond to Kn and n respectively. Hence from the two lines K and n values for the two classes of binding site are determined. The curve fitting methodology and the positioning of

tangents may drastically alter the values of K and n determined by orders of magnitude. Where a large degree of scatter is observed the numerical data generated by this procedure may be dubious.

Figure 6.3 A schematic representation of typical curvature observed applying Scatchard analysis to binding data.



Indeed, Klotz and Hunston (1979) have stated that there is no basis for such an interpretation and that curvature is commonly a manifestation of interactions between initially identical binding sites. It may alternatively represent the presence of numerous different binding sites. When considering the complexity and heterogeneity of mucus this latter theory may be more appropriate. For these reasons the n and k values were not determined from the Scatchard plots in these experiments.

6.3 EXPERIMENTAL

The binding experiments were carried out according to the centrifugation method of Saitoh et al. (1986). Stock solutions of 0.05M and 1mM of the drug to be investigated were prepared in 0.1M phosphate buffer, pH 7.4. Dilutions of these stock solutions were made to produce eleven solutions of the required concentration ranging from 0.05M - 5 μM. Mucus (0.4g) was weighed directly into centrifuge tubes. To this 4 mL of the drug solution was added and the tubes vortexed. The mixture was then incubated for 30 minutes at 20°C in a shaking water bath. To separate the free from the mucus-bound drug the mixture was centrifuged at 29,000 x *g* for 30 min at 4°C in a Europa 24M centrifuge. Two millilitres of the supernatant were then removed and the drug concentration was determined by reversed-phase HPLC, as described in section 2.3.1.

Control experiments with the same mass of mucus and drug-free phosphate buffer were carried out to ensure that there was no interference with the HPLC assays from any mucus components. An additional control was carried out to establish whether drug molecules were lost to non-mucus sources during the experiments. This was determined by same procedure as described above for mucus-binding, but in the absence of mucus.

The compounds evaluated, to assess the effect of molecular weight and chemical functional group, were the amino acid TRP, the dipeptide WG, the tripeptide WGG and the amide of phenylalanine NP. The carboxylic acid BA and the three β-blockers PR, OX and AT were investigated to assess the effect of electrical charge and lipophilicity.

6.4 RESULTS AND DISCUSSION

The extent of drug binding to mucus is important in determining the quantity of free drug available for diffusion and may impair the absorption of the drug. In these studies, all of the compounds tested, with the exception of BA, bound to mucus to a greater or lesser extent. The numerical data determined for the percentage of drug bound are presented in tables 6.1, 6.2 and 6.3.

Table 6.1 Binding data for tryptophan (TRP), tryptophylglycine (WG) and tryptophylglycylglycine (WGG) to 1% w/v pig gastric mucus. Values are the mean ± $S.D_{n-1}$ of two determinations. D_t, is the total drug concentration and NT indicates that the binding was not tested at this drug concentration.

D_t (M)	TRP (%)	WG (%)	WGG (%)
0.05	NT	NT	3.99 ± 0.16
0.025	1.00 ± 1.40	1.68 ± 1.08	NT
0.01	4.88 ± 1.71	4.51 ± 1.30	2.66 ± 0.00
5 x 10^{-3}	3.41 ± 0.43	6.55 ± 2.69	1.26 ± 0.76
1 x 10^{-3}	0.98 ± 0.84	17.82 ± 6.31	10.9 ± 6.42
5 x 10^{-4}	4.23 ± 1.64	6.72 ± 6.88	4.93 ± 0.80
2.5 x 10^{-4}	2.76 ± 0.84	1.11 ± 0.00	6.97 ± 0.90
1 x 10^{-4}	6.04 ± 7.17	4.87 ± 3.18	17.75 ± 0.63
7.5 x 10^{-5}	3.46 ± 3.00	12.82 ± 13.06	7.90 ± 0.48
5 x 10^{-5}	11.67 ± 4.54	2.28 ± 11.04	2.15 ± 4.41
2.5 x 10^{-5}	1.43 ± 0.82	17.52 ± 9.30	4.37 ± 4.27
1 x 10^{-5}	NT	50.99 ± 6.66	21.14 ± 1.77
5 x 10^{-6}	NT	NT	36.13 ± 0.00

Table 6.2 Binding data for N-acetylphenylalanine (NP) to 1% w/v pig gastric mucus. Values are the mean ± $S.D_{n-1}$ of two determinations. D_t, is the total drug concentration.

D_t (M)	NP (%)
0.05	3.89 ± 2.27
0.025	0.58 ± 2.47
0.01	1.45 ± 0.41
5 x 10^{-3}	0.52 ± 0.00
1 x 10^{-3}	1.47 ± 0.70
5 x 10^{-4}	3.70 ± 0.87
2.5 x 10^{-4}	2.77 ± 0.00
1 x 10^{-4}	5.43 ± 2.56
7.5 x 10^{-5}	19.35 ± 6.70
5 x 10^{-5}	17.82 ± 0.00
2.5 x 10^{-5}	38.30 ± 0.00

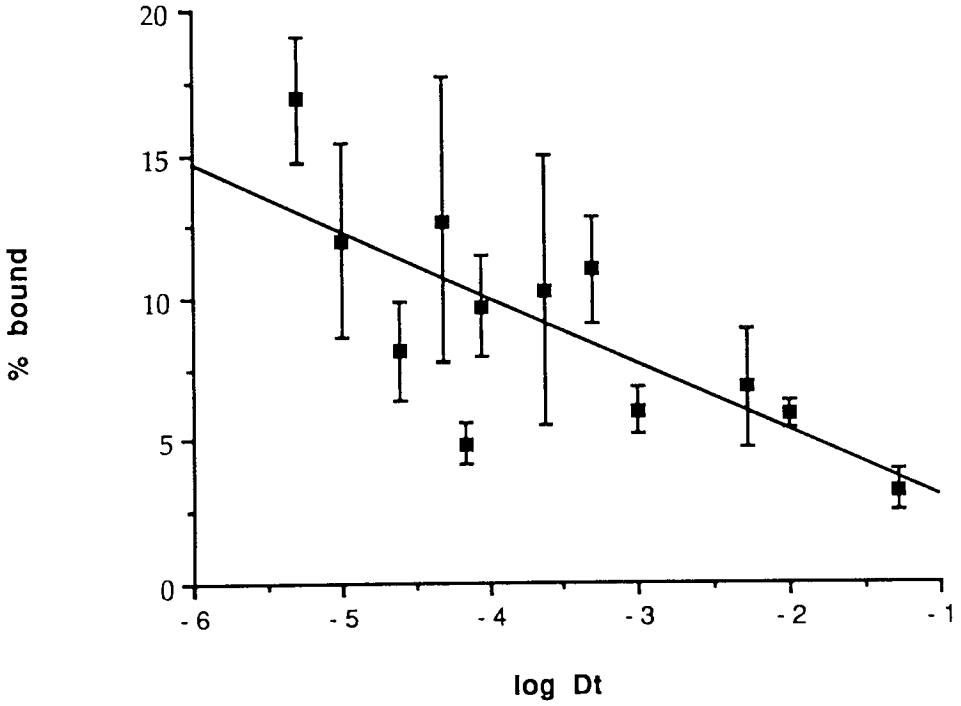
Table 6.3 Binding data for propranolol (PR), oxprenolol (OX) and atenolol (AT) to 1% w/v pig gastric mucus. Values are the mean of two determinations ± SD_{n-1}. D_t, is the total drug concentration and NT indicates that the binding was not tested at this drug concentration.

D_t (M)	PR (%)	OX (%)	AT (%)
0.05	3.25 ± 0.77	NT	NT
0.025	NT	4.10 ± 0.44	1.47 ± 2.08
0.01	5.93 ± 0.48	5.14 ± 0.00	7.13 ± 4.32
5×10^{-3}	6.85 ± 2.08	2.54 ± 1.79	12.36 ± 0.63
1×10^{-3}	6.04 ± 0.82	4.96 ± 1.40	4.00 ± 2.83
5×10^{-4}	11.03 ± 1.89	2.15 ± 1.01	2.92 ± 4.14
2.5×10^{-4}	10.30 ± 4.77	1.36 ± 1.92	12.74 ± 5.54
1×10^{-4}	9.75 ± 1.82	1.48 ± 1.05	1.11 ± 3.14
7.5×10^{-5}	4.87 ± 0.74	3.43 ± 0.69	0.72 ± 0
5×10^{-5}	12.76 ± 9.66	0.60 ± 0.85	2.00 ± 2.83
2.5×10^{-5}	8.15 ± 1.77	11.78 ± 0.00	4.00 ± 0
1×10^{-5}	12.01 ± 3.40	NT	15.77 ± 0
5×10^{-6}	16.95 ± 2.18	NT	NT

The percentage of drug bound, for each compound, was greatest at the lowest concentration of total drug (D_t) and least at the highest value of D_t. This suggested that as the concentration of total drug (D_t) was increased, the percentage of bound drug also increased. Between these two values however, the percentage bound exhibited a more variable, somewhat random distribution, which made comparison between the different compounds difficult. A similar result was reported by Hughes (1988) for the binding of several compounds to purified gastric mucus. The percentage bound was never greater than 50% for any compound tested and below 50 μM the percentage bound was less than 18%. It suggested that none of the compounds evaluated bound to mucus in appreciable amounts.

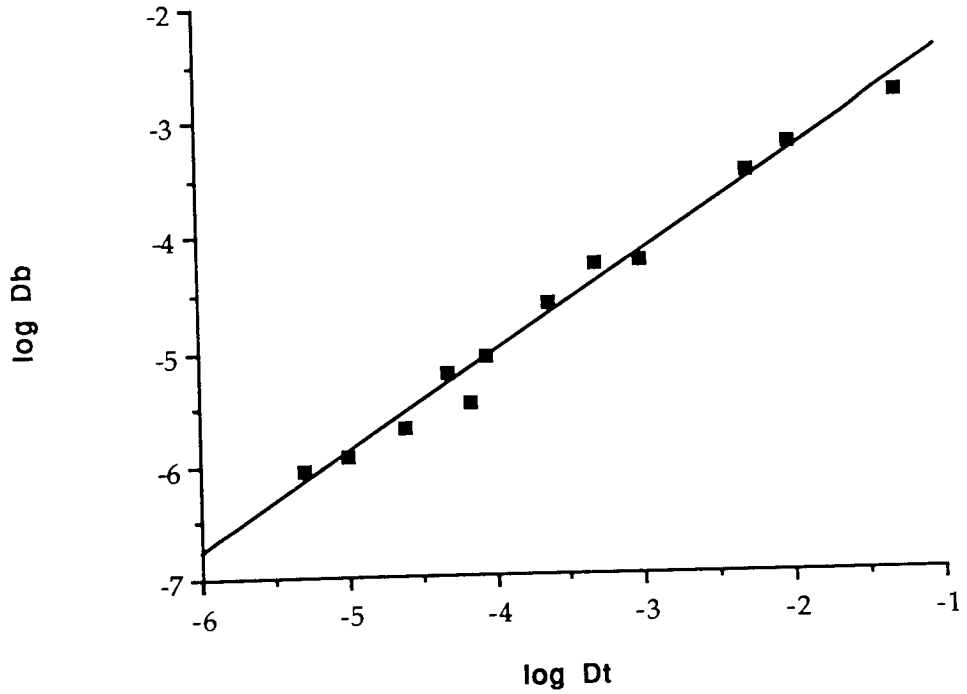
Plots of log D_t versus the percentage of drug bound did however indicate a linear trend. The binding of PR to mucus in this format, is illustrated in figure 6.4, as an example.

Figure 6.4 The binding of propranolol to pig gastric mucus. The results are expressed as log the total drug concentration (D_t) versus the percentage of drug bound. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Points are the mean ± sem of two determinations.



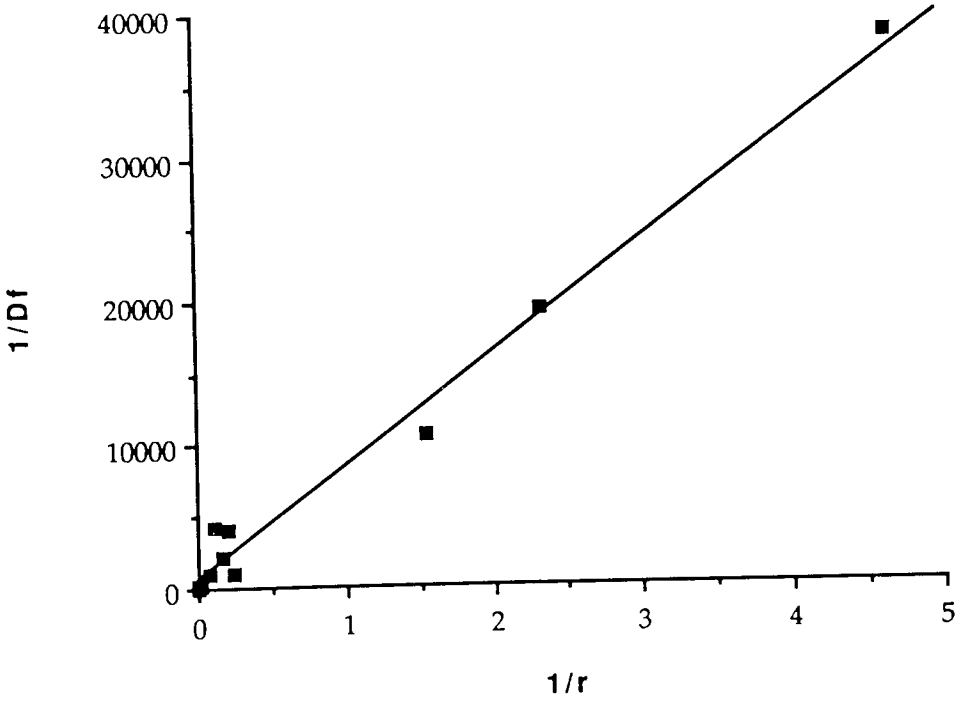
A linear trend could also be produced from a plot of log (D_t) versus the log concentration bound (D_b). The binding of PR to mucus is illustrated in figure 6.5. The correlation coefficient was 0.993.

Figure 6.5 The binding of propranolol to pig gastric mucus of log the total drug concentration (D_t) versus log the concentration of drug bound (D_b). Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Each points is a single determination.



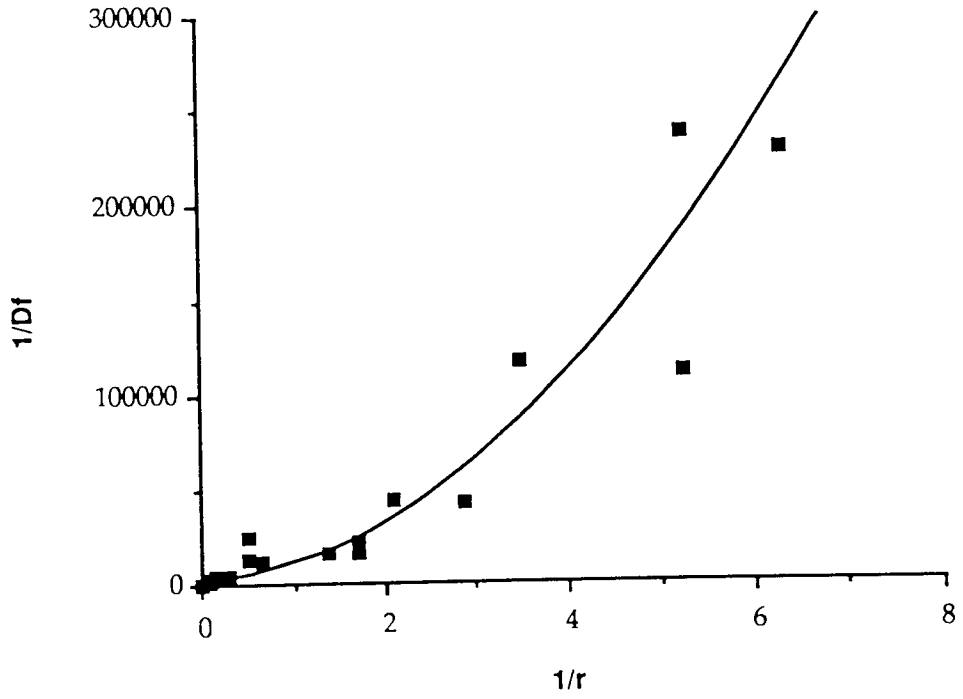
The binding of each of the seven compounds which associated with the mucus, was analysed by constructing Klotz, Scatchard and Freundlich plots as described in section 6.2. Of the seven compounds analysed by this methodology, only one, atenolol (AT) produced a linear plot (r = 0.998), indicating the presence of one type of non-interacting binding site. This is illustrated in figure 6.6.

Figure 6.6 Klotz plot for the binding of atenolol to pig gastric mucus. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Each point is a single determination.



The Klotz plots for the binding of the other compounds were non-linear. An example of the typical non-linear, concave curve is illustrated in figure 6.7 for PR. Similar plots were reported by Hughes (1988) for the binding of a variety of compounds to purified pig gastric mucus.

Figure 6.7 Klotz plot for the binding of propranolol to pig gastric mucus. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Each point is a single determination.

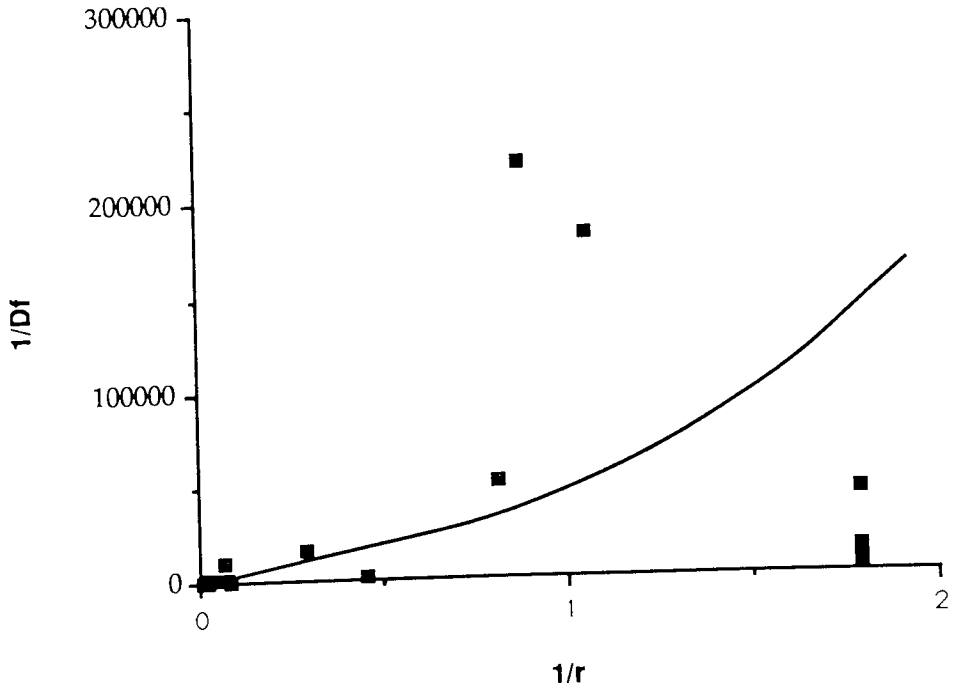


The non-linearity may be due to the presence of more than one binding site for these compounds or, alternatively, may reflect inter-binding site interactions which render the one type of binding site non-equivalent. Klotz et al. (1946) reported pronounced convex curvature

in the Klotz plot for the binding of azosulphathaizole to bovine serum albumin. They proposed that each anion added to the protein exerted an additional electrostatic repulsion toward further oncoming ions. This consequently decreased the relative extent of binding. The non-linearity may be due to this or other factors, such as adsorption.

Varying degrees of scatter were evident in the Klotz plots. Two of compounds tested, TRP and WG, exhibited a high degree of scatter. The Klotz plot for the binding of WG to mucus is illustrated in figure 6.8.

Figure 6.8 A Klotz plot for the binding of tryptophylglycine to pig gastric mucus. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Each point is a single determination.



The scatter observed in Klotz plots may be due to differences in the individual mucus samples used in the experiments, which would be compounded by the small mass of mucus used. Such an effect would, however, be unexpected from the repeatability and reproducibility results for the diffusion through mucus in section 4.4.1, where the diffusion rates through different batches of mucus were not significantly different ($p < 0.05$).

Linear Scatchard binding plots, like linear Klotz plots, are proposed to demonstrate the presence of one type of non-interacting binding site. Scatchard analysis for the binding of each compound to mucus produced concave curves similar to the schematic representation in figure 6.3. This result was in agreement with the results for the Klotz analysis. The presence of more than one binding site within the mucus gel would be unsurprising in view of the complexity of the secretion. The Scatchard plot for the binding of PR to mucus typifies plots obtained in this study and is presented in figure 6.9.

The curves obtained in the Scatchard plots were typical of those reported by a number of workers for the binding of molecules to mucus. These include positively-charged ions (Crowther and Marriott, 1984), tetracycline (Kearney and Marriott, 1987), EDTA and DTPA (Cheema et al., 1988) and a series of compounds with different physicochemical properties (Hughes, 1988), over a range of total drug concentrations. A line was drawn through the experimental data points to demonstrate the concave distribution of points. Scatter from this assigned line was observed which in most cases was comparable to that reported for binding to mucus and binding to bovine serum albumin (Rowland and Tucker, 1986). An example where a larger degree of variation was observed is shown in figure 6.10, for OX.

Figure 6.9 Scatchard plot for the binding of propranolol to pig gastric mucus. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Each point is a single determination.

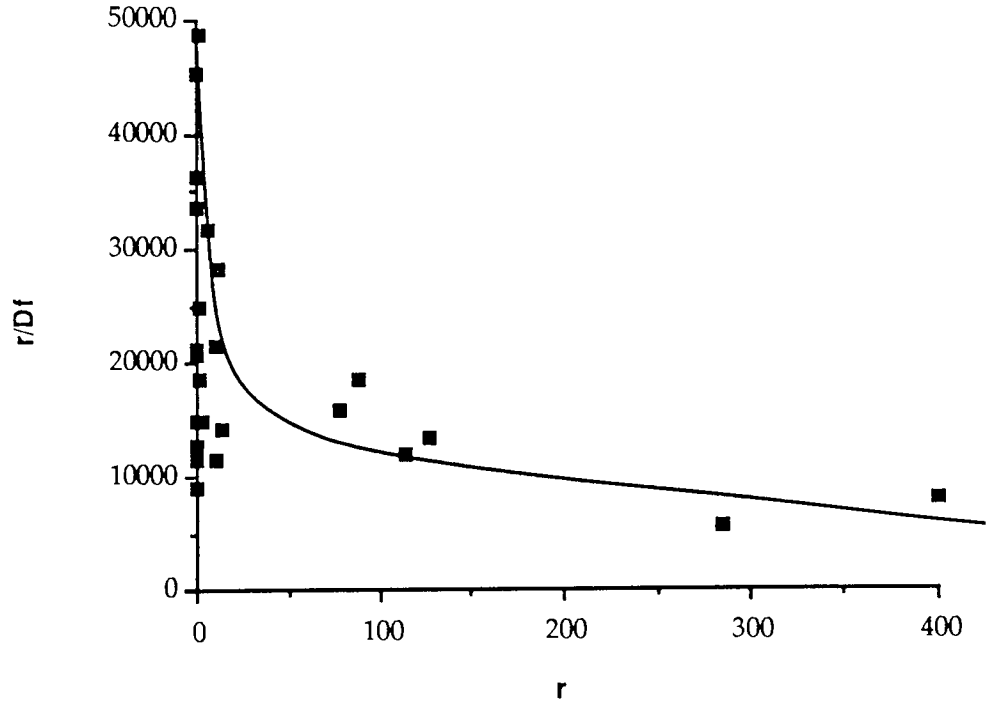
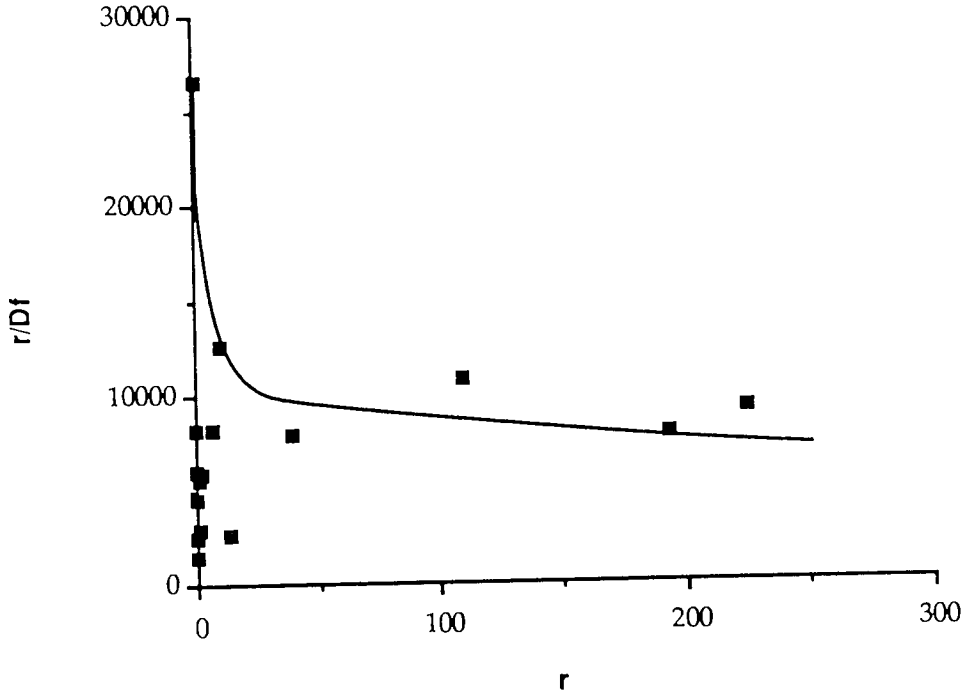
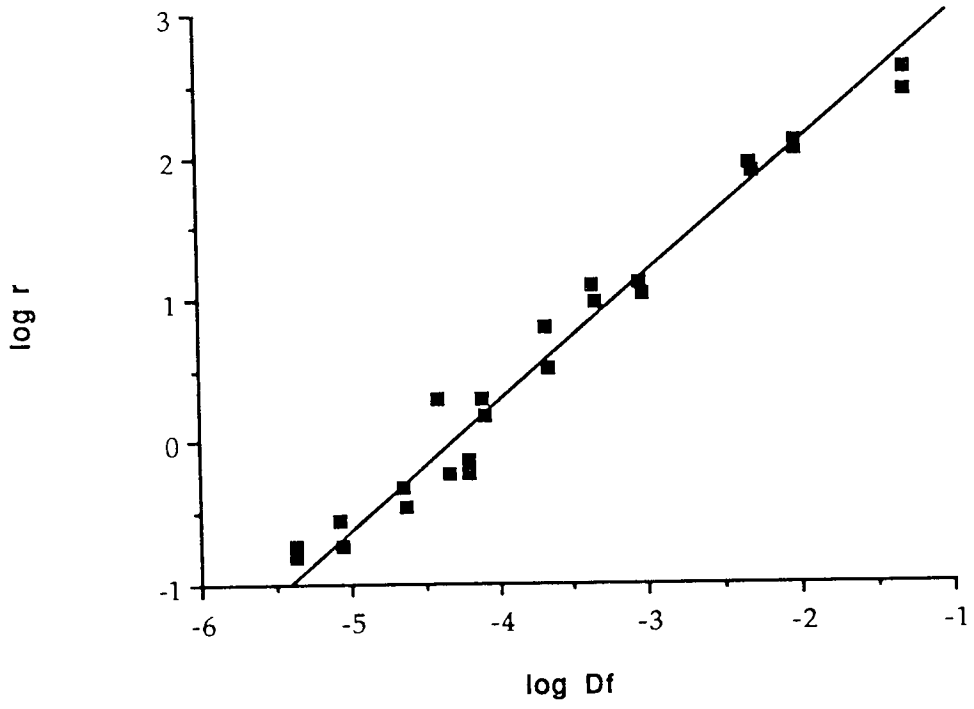


Figure 6.10 Scatchard plot for the binding of oxprenolol to pig gastric mucus. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Each point is a single determination.



The third type of analysis utilised the Freundlich adsorption isotherm, where log r is plotted versus log D_f. An assumption in the derivation of the Freundlich equation is that the surface of the adsorbent is heterogeneous. Linear plots were obtained for all the compounds tested, which suggested that the mucus offered non-equivalent binding sites, in agreement with the Klotz and Scatchard results. A typical example of this behaviour is illustrated in figure 6.11 for PR, which had a linear correlation coefficient of 0.989

Figure 6.11 Freundlich plot for the binding of propranolol to pig gastric mucus. Experiments were performed in phosphate buffer (pH 7.4) at 20°C. Each point is a single determination.



The Freundlich parameters were determined for each compound from the line of best fit through the experimental data, determined by linear regression. The values are presented in table 6.4. Adamson (1990) reported that the product of the parameters a and n was a measure of the adsorbent capacity, and 1/n a measure of the intensity. These values are also presented in table 6.4

Table 6.4 Freundlich parameters determined for all compounds tested.

compound	a	n	a.n	1/n	correlation coefficient
TRP	6006	0.988	5934	1.012	0.954
WG	342	1.998	683	0.501	0.716
WGG	1284	1.478	1898	0.677	0.936
NP	589	1.599	942	0.625	0.847
PR	8525	1.092	9309	0.916	0.989
OX	6437	0.993	6392	1.007	0.941
AT	8829	1.026	9059	0.975	0.941

The mechanism of binding to mucus for the compounds tested may be by electrostatic and possibly hydrophobic interactions. The positively charged amino groups (e.g. in TRP, WG and WGG), may interact with the negatively charged mucus residues, and aromatic rings may additionally be a centre for hydrophobic binding. It is acknowledged that both ionic and hydrophobic forces are involved in protein binding and that the relative contribution of the two depends on the chemical series under evaluation (Rowland and Tucker, 1986). The adsorbent capacity of the series TRP, WG and WGG, was TRP (5934) > WGG (1898) > WG (683) and the intensity had the same rank order. This suggested that TRP underwent greater and stronger binding to mucus than the other two compounds.

By blocking the positively charged amino group of PHE to produce NP it was postulated that the electrostatic interactions between the compound and mucus would be reduced. Therefore, if this were the major mechanism of binding the quantity of bound drug would be

correspondingly reduced. This was suggested by the observation that no interactions occurred between purified gastric mucus and either the neutral molecule caffeine or the negatively charged salicylic acid (Hughes, 1988), implicating the importance of charge in binding, and is consistent with the lack of binding observed for BA in this study. For NP, both the product of a and n (942), and the 1/n value (0.625) were considerably smaller than those determined for TRP, which suggested that this compound was less adsorbed onto mucus than TRP. Saitoh et al. (1986) were however unable to relate the degree of ionization of tertiary amines with their binding to mucus. In view of this observation they proposed that the differences in binding were due to the differing hydrophobicities of the compounds.

The three β-blockers PR, OX and AT are all positively charged at pH 7.4. They are all bound to mucus in comparison with the negatively charged carboxylic acid BA. The compounds PR, OX and AT have similar pKa values (9.23, 9.32 and 9.32 respectively) but have a range of lipophilicities, with PR being very lipophilic (Log P = 3.21), OX lipophilic (Log P = 2.37) and AT hydrophilic (log P = 0.16). Any difference in the binding of these three compounds might therefore be due to hydrophobic interactions. The product of a and n for these compounds was PR (9309) > AT (9059) > OX (6392), whilst the order of 1/n was reversed with OX (1.007) > AT (0.975) > PR (0.916). The adsorbent capacity implied that the most lipophilic compound, PR, may be bound to a greater extent than the other two compounds. Increased binding could be due to hydrophobic interactions with either the protein domain of the glycoprotein (Pearson et al., 1981), with lipids associated with the mucus gel or with covalently-bound lipids (Slomiany et al., 1988). It is impossible from this limited data to state how increasing hydrophobicity might affect binding, especially as the adsorbent capacity would suggest very lipophilic > hydrophilic > lipophilic. Studies investigating the binding of a large series of sulphonylurea-related compounds with the protein bovine serum albumin have observed a positive correlation between increasing hydrophobicity (expressed as octanol/water partition coefficients) and increased binding (Goto et al., 1978). A similar trend was observed for 2- and

4-sulphapyrimidines binding to bovine serum albumin (Rowland and Tucker, 1986).

Such a trend may be observed for drugs binding to mucus if a larger series of compounds were evaluated. The importance of hydrophobic binding may account for the high binding results obtained for the very lipophilic compound testosterone (log P = 3.32) where more than 40 % was bound to purified gastric mucus (Hughes, 1988)

In the diffusion experiments described in section 4.4.3 it was demonstrated that the negatively charged compound BA diffused through mucus the most rapidly (ratio = 76.19 ± 0.18 %) of the eight compounds evaluated. Additionally, this compound does not appear to bind to mucus, which may be a result of electrostatic repulsion from the negatively charged sialic and sulphated groups the glycoprotein molecules. The positively charged β-blockers PR, OX and AT, however, diffused through mucus significantly slower than BA (ratio = 55.50 ± 5.65 %, 51.58 ± 2.91 % and 46.23 ± 3.67 % respectively), and were all found to interact with mucus ($p < 0.05$). This suggests that retention of a compound by the mucus network may be negatively correlated with diffusion through the mucus barrier. A similar correlation has been reported for the binding to, and diffusion through unpurified rat intestinal mucus by a series of ergotamine derivatives (Nimmerfall and Rosenthaler, 1980) and some antibiotics using hog gastric mucin (Saggers and Lawson, 1966). The strong binding of cationic aminoglycosides to purified cystic fibrosis sputum has also been proposed as an important predictor of their *in vivo* activity (Ramphal et al., 1988).

The diffusion of the tripeptide WGG was most retarded by imposition of a mucus layer (ratio = 40.94 ± 3.80 %) which was a significantly greater reduction ($p < 0.05$) than that observed for the smaller amino acid TRP (ratio = 55.12 ± 5.94). The binding capacity and intensity of binding for WGG appeared to be of smaller magnitude than for TRP. This suggests that the different diffusivities for these three compounds may be due to a mechanical obstructive effect by mucus based

on increasing molecular weight, rather than due to a chemical interaction with the constituents of the mucus gel. This may not be the case for all peptide-based molecules and it is possible that larger peptidic compounds containing more hydrophobic residues may bind strongly to mucus, which would affect systemic absorption.

6.5 SUMMARY

A method of centrifugation was employed to investigate the binding of eight model compounds TRP, WG, WGG, NP, BA, PR, OX and AT to pig gastric mucus. All of the compounds evaluated bound to mucus to some extent with the exception of BA. The lack of association for BA was proposed to be due to electrostatic repulsion between the drug molecule and the negatively charged groups on the glycoprotein molecules.

The Klotz, Scatchard and Freundlich plots indicated either the presence of more than one binding site, or that there were interactions between the binding sites rendering the one class of site no longer equivalent.A measure of the adsorbent capacity of mucus and the intensity of binding was determined from the Freundlich parameters. The three positively charged β-blockers, PR, OX and AT, had the greatest adsorbent capacities and intensity of binding. This implied that electrostatic interactions with mucus were an important factor in drug-mucus binding. The adsorbent capacity of NP, where the positively charged amino group was blocked, was appreciably smaller than that for TRP, with an amino group. Hydrophobic interactions may also be important in drug-binding to mucus, although the adsorbent capacities and intensity of binding of the hydrophilic compound AT, and the very lipophilic compound PR were of similar magnitudes.

The relationship between binding and diffusion for BA and the β-blockers suggested a negative correlation between binding and diffusion. In contrast, the compounds WGG and NP, which diffused more slowly than TRP, exhibited smaller adsorbent capacities and weaker binding intensities.

CHAPTER 7
HYDROLYSIS OF PEPTIDES.

CHAPTER 7

HYDROLYSIS OF PEPTIDES.

7.1 INTRODUCTION

The unsuccessful oral delivery of most peptide and protein drugs is largely attributable to the numerous proteolytic enzymes in the GI tract, which hydrolyse the molecules and render them biologically inactive (Lee, 1986). It has also been established that degradation can occur luminally, at the brush border and intracellularly. Moreover, the site of degradation is dependent upon the size and amino acid composition of the peptide (Lee, 1988; Lee and Yamamoto, 1990). The proteolytic activities, subcellular distribution and substrate specificities of enzymes at other potential sites of drug administration (e.g. the nose) are, however, less well characterised and are currently areas of active research.

Although it was originally believed that the nasal mucosa was deficient in enzymic activity, it has more recently been shown from *in vitro* metabolism studies that the nasal mucosa possesses a large armoury of enzymes. Those characterised to date include: monooxygenases (Longo et al., 1986, 1989); reductases (Llarsson at al., 1989); transferases (Aceto et al., 1989); esterases (Zhou and Li Wan Po, 1990, 1991) and a variety of proteases (Dodda Kashi and Lee, 1986; Stratford and Lee, 1986).

A large proportion of the work has been carried out to determine the metabolic capacity of the nose, with respect to the ability of nasal tissue to activate or detoxify carcinogenic compounds implicated in nasal and respiratory cancer. These include aflotoxin B (Llarsson et al., 1989), nitrosamines (Brittebo et al., 1983), polycyclic amines (Bond, 1983) and benza(a)pyrene (Matsubara et al., 1974). These experiments, and others, have demonstrated the presence of cytochrome P-450-dependent monooxygenase in rat, hamster (Longo et al., 1986) and human nasal microsomes (Longo et al., 1989). Moreover, the concentrations of the cytochrome P-450 are second only to that of the liver as determined by the activity per milligram of microsomal protein (Dahl et al., 1982).

Significant levels of esterase activity have been determined in rat, rabbit and guinea-pig nasal mucosa, as demonstrated by the ability of homogenate preparations to generate p-nitrophenol from p-nitrophenyl acetate (Zhou and Li Wan Po, 1991). The nose has also been shown to be a site of steroid metabolism for hormones such as oestradiol, progesterone and testosterone (Barwashi-Nassar et al., 1989a and b).

It was the disappointing results for the absorption of peptides from the nasal cavity which prompted the investigation of the proteolytic activity of the nose, with *in vivo* (Hussain et al., 1985) and *in vitro* (Hirai et al., 1981a and b) methodology. Metabolism of a variety of peptide and protein substrates has confirmed the presence of both endopeptidases and exopeptidases. The use of enzyme-specific substrates, enzyme activators and inhibitors (summarised in table 7.1), has shown specific enzymes present to include aminopeptidases A, B, N, leucine aminopeptidase and microsomal aminopeptidase (Stratford and Lee, 1986). Similarly, the pattern of peptide degradation has suggested the presence of aminopeptidases, dipeptidylpeptidase, diaminopeptidase, post-prolyl cleaving-enzyme, angiotensin converting-enzyme and endopeptidase EC 24.11 (Dodda Kashi and Lee, 1986; Lee et al., 1987; Hayakawa et al., 1987).

The subcellular activity has been probed by differential centrifugation of tissue homogenates, using either enkephalins or insulin as substrates. The activity against the three enkephalins tested was resident in both the plasma and cytosolic fraction, with 48-54% activity residing in the plasma fraction (Choi and Lee, 1986). Using insulin as a substrate, however, 79% of the proteolytic activity resided in the membrane fraction, which was very similar to the distribution of activity in the ileum (82% membrane bound) (Yamamoto et al., 1988).

Table 7.1 A summary of chemical substrates, activators and inhibitors (bestatin and puromycin) used to determine aminopeptidase activity. The substrates were 4-methoxy-2-naphthylamides of L-leucine, L-alanine, L-arginine and L-glutamic acid (Stratford and Lee, 1986).

Amino peptidase	substrate name	structure (R)	activator	inhibitor
leucine	L-leucine	$CH_2CH(CH_3)_2$	0.1mM Mn^{2+}	bestatin
N	L-alanine	CH_3	0.1mM Co^{2+}	both
lysosomal	L-arginine	$(CH_2)_3NHC(NH_2)=NH$	0.1mM dithiothreitol	not stated
B	L-arginine	$(CH_2)_3NHC(NH_2)=NH$	0.1M Cl-	both
A	L-glutamic acid	$(CH_2)_2COO-$	10mM Ca^{2+}	neither

The proteolytic activity of the nasal route, relative to that of the other delivery routes is open to debate. Dodda Kashi and Lee (1986) concluded from their studies with mucosal tissue from the albino rabbit, that with enkephalin substrates the order of activities was rectal > nasal > buccal > vaginal. In contrast, with insulin and proinsulin as substrates and using the same animal model, the order became rectal > vaginal > nasal > buccal (Lee et al., 1987). A more recent study investigating aminopeptidase activity in rat mucosal tissue showed that the order of activity was intestinal > rectal > buccal > nasal > dermal (Zhou and Li Wan Po, 1990). These differences may be due to enzymes responsible for degrading particular substrates being more abundant at some mucosal sites than others.

A number of animal species have been used for *in vivo* studies of nasal drug delivery. Those most commonly employed are dogs, rats, rabbits and sheep (Chien et al., 1989) and the relevance of the different models to the human situation and anatomical and physiological variations was recently reviewed by Gizurarson (1990). The report suggested that small animals such as guinea-pigs, hamsters, rats and mice, whilst being inexpensive and easy to handle were unsuitable for formulation studies and pharmacokinetic profiles, but

could be useful for absorption and absorption enhancing studies. The dog, monkey, sheep and rabbit were considered to be particularly useful for pharmacokinetic and formulation studies.

In the present study, sheep nasal mucosa was employed for several reasons. Firstly, it has been established as a model *in vivo* (Farraj et al., 1990) and *in vitro* (Wheatley et al., 1988) for nasal delivery, and good correlation between the intranasal absorption of insulin in man and sheep has been demonstrated (Longenecker et al., 1987). Secondly, it was possible to obtain viable tissue from a local abattoir, hence eliminating the need to sacrifice laboratory animals. Finally, it was hoped to establish an *in vitro* model for the nasal mucosa based on Ussing chambers and sheep tissue. It therefore seemed prudent to use the same animal species for both experimental procedures, to allow direct comparison of results.

7.2 THEORETICAL TREATMENT OF RESULTS

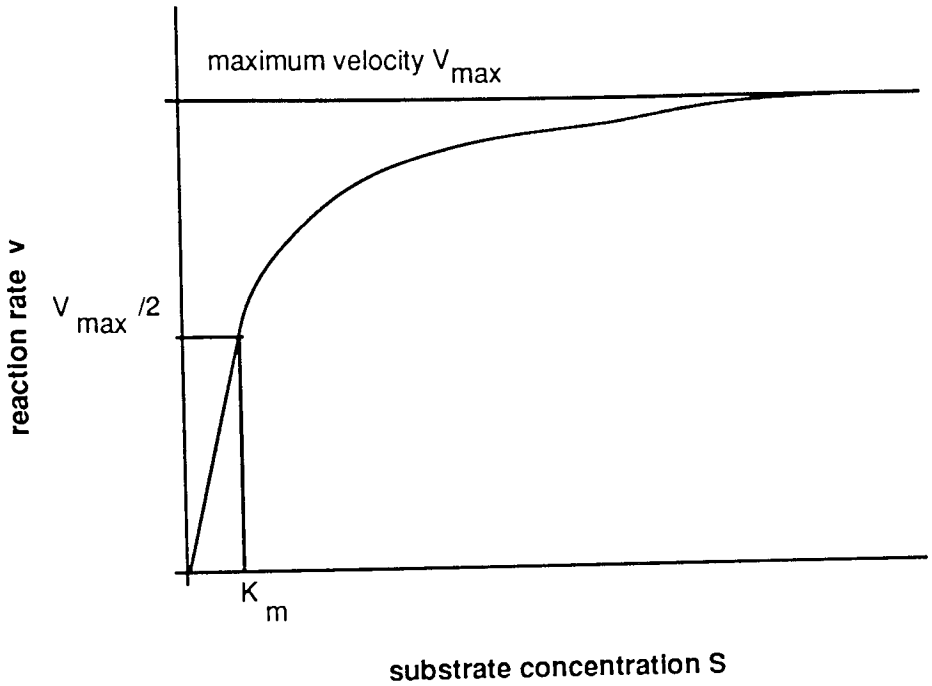
The rate of an enzyme-catalysed reaction is dependent upon the concentration of both enzyme and substrate. If the concentration of substrate is in excess, the reaction rate increases linearly with increasing enzyme concentration. For a fixed concentration of enzyme however, increasing the substrate concentration will increase the rate of the reaction in a non-linear relationship until the maximum rate occurs. Increasing the substrate concentration above this will not increase the rate further. This relationship is described by the Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{[S] + K_m} \qquad (7.1)$$

The term v is the rate of the reaction at a given time, V_{max} is the maximum reaction rate and [S] is the substrate concentration. The term K_m is the Michaelis constant, which is equal to the substrate concentration at which the reaction rate is half its maximum value.

This relationship is illustrated graphically in figure 7.1.

Figure 7.1 A schematic representation of typical enzyme kinetics.



In enzyme studies the terms V_{max} and K_m are determined to give an indication of any preference an enzyme may have for a particular substrate. Classically, the kinetic parameters are determined by performing a series of metabolism studies employing the same enzyme concentration and a range of substrate concentrations. Determination of the initial rates of reaction permits reciprocal plots of equation 7.1 to be drawn, and V_{max} and K_m determined from the appropriate slope and intercept of the resultant line. An example of this approach is the Lineweaver-Burk double reciprocal transformation where:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \qquad (7.2)$$

and a plot of 1/v versus 1/[S] permits the determination of V_{max} and K_m from the intercept and slope of the line of best fit through the

data. An alternative method of determining V_{max} and K_m is the method described by Nichols and Hewinson (1987), which is based on the theoretical work on half-time analysis by Wharton and Szawelski (1982).

The basis of the method is the integration of the Michaelis-Menten equation (7.1) to yield equation 7.3.

$$\ln(S_t) + \frac{S_t}{K_m} = \ln(S_o) + \frac{S_o}{K_m} - \frac{V_{max}t}{K_m} \qquad (7.3)$$

This non-linear equation can be arranged to produce a linear relationship between $t_{½}$ and S_o when $S_t = 0.5S_o$:

$$t_{½} = \frac{S_o}{2V_{max}} + \frac{K_m \ln 2}{V_{max}} \qquad (7.4)$$

The term S_o is the local substrate concentration at any time on the reaction progress curve and $t_{1/2}$, is the time taken for any S_o to fall to $S_o/2$.

The procedure is, therefore, to record the complete hydrolysis profile of a substrate, and, using this progress curve, to measure $t_{½}$ for several different values of S_o. By plotting $t_{½}$ versus S_o graphically, it is then possible to determine the value of V_{max} from the gradient of the line ($1/2V_{max}$) and subsequently, to determine K_m from the intercept on the ordinate axis. At this point, where S_o is equal to zero, the intercept will have a value corresponding to $K_m \ln 2/V_{max}$. The model requires the complete progress curve and assumes that the products exert no effect on the rate of the reaction. Additionally, to obtain reliable enzyme kinetics the initial concentration of S_o should be $1\text{-}3xK_m$ ($0.5\text{-}5xK_m$ is usable).

The validity of these determined values may be determined by sequential computer modelling of the Michaelis-Menten equation (7.1) and comparison of the theoretical and experimental profiles.

In relation to chemical kinetics, the Michaelis-Menten equation (7.1) can be seen to approximate to both zero order and first order reactions, depending upon the concentration of the substrate. If the substrate concentration is considerably larger than the K_m value equation 7.1 will reduce to:

$$v = V_{max} \tag{7.5}$$

which would be a zero order reaction, independent of [S]. If, however the substrate concentration was considerably smaller than K_m, equation 7.1 would approximate to:

$$v = \frac{V_{max}[S]}{K_m} \tag{7.6}$$

Under these conditions, the reaction would appear to be a first order reaction, where the rate of reaction depends upon substrate concentration only, and v is equivalent to k[S].

First order rate constants (k) can be estimated from semilogarithmic plots of concentration versus time. The slope, of the best line of fit, determined by linear regression, yields k. The half-life $t_{½}$ can then be determined from the following equation:

$$t_{½} = \frac{0.693}{k} \tag{7.7}$$

where $t_{½}$ is the time taken for half the initial concentration of substrate to have disappeared.

The foregoing refers only to the disappearance of substrate. When degradation products undergo further reaction it is also possible to model such processes. For example, the hydrolysis of substance A, may be regarded as a two-stage first order sequential reaction, in which the initial component A degrades to substance B which further reacts

to yield substance C. This can be represented by the following reaction scheme:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

In time all of component A is converted to C, with the concentration of B rising at first and then decreasing to zero. It is therefore possible to construct theoretical progress curves of each substrate. The equations which model this system are:

$$A_t = A_0 . e^{-k_1 t} \qquad (7.8)$$

$$B_t = \frac{k_1 . A_0}{k_2 - k_1} . (e^{-k_1 t} - e^{-k_2 t}) \qquad (7.9)$$

$$C_t = A_0 - \frac{A_0}{k_2 - k_1} . (k_2 . e^{-k_1 t} - k_1 . e^{-k_2 t}) \qquad (7.10)$$

where A_t, B_t and C_t are the concentrations of components A, B and C respectively at time t and A_0 is the initial concentration of component A. The terms k_1 and k_2 are the first-order rate constants for the conversion of components A to B and B to C respectively. The rate constants may be determined by iteration using the computer program NONREG (Irwin, 1990) which uses a non-linear least squares regression analysis.

7.3 EXPERIMENTAL

7.3.1 Enzymic Preparations

7.3.1.1 Preparation of nasal homogenate

The nasal homogenate was prepared by a modified method of Stratford and Lee (1986). The heads of recently slaughtered sheep were obtained from a local abattoir. The heads were bisected along the nasal septum to expose the nasal cavity. The nasal mucosa was carefully excised from the nasal septum and turbinates and freed from the underlying cartilage and bone. The tissue was rinsed with 0.1M phosphate buffer, pooled in glass vials and stored at -70°C.

Prior to use, the tissue was thawed at room temperature for about 30 min. Phosphate buffer was added to the tissue to produce a 100% (w/v) mixture. The tissue was homogenized in an Omnimixer for 2 min at 4°C then in a Teflon-glass homogeniser. The homogenate was than centrifuged at 3020 *g* at 4°C for 10 min to remove cellular and nuclear debris. The supernatant produced, which contained both cytosolic, plasma and intracellular membrane fractions was diluted in phosphate buffer and equilibrated at 37°C for 30 min then used immediately.

To determine the effect of pH upon the hydrolysis, McIlvaine's buffer (pH 7.4) was employed to prepare the homogenate. The composition of this buffer is given in appendix 2.

7.3.1.2 Preparation of microsomes

Microsomal and cytosolic fractions were prepared by a modified method of Llarsson et al. (1989). The nasal homogenate was prepared as described above except McIlvaine's buffer (pH 7.4) was used in place of 0.1M phosphate buffer with added KCl, as detailed in appendix 2. To separate the cytosolic and microsomal fractions the homogenate was centrifuged in a Beckman model TL-100 ultracentrifuge at 100,000g for 1 h at 4°C. After this the supernatant, which represented the cytosolic fraction was removed. The microsomal pellet was resuspended in McIlvaine's buffer (pH 7.4) with a Teflon homogeniser. The cytosolic and microsomal fractions were diluted with McIlvaine's buffer and equilibrated at 37°C for 30 min.

7.3.1.3 Experiments performed with pure enzymes

Three commercially available proteolytic enzymes were employed in light of the aminopeptidase and carboxypeptidase activities exhibited by the homogenate preparation. These comprised carboxypeptidase A, and cytosolic and microsomal leucine aminopeptidase. Each of the enzyme preparations was diluted in 0.1M phosphate buffer (pH 7.4) to give a final protein concentration of 10 μg mL^{-1} in the incubation mixture. The enzymes were equilibrated at 37°C in a water bath for 30 min before the metabolism experiment was initiated by addition of the substrate.

Carboxypeptidase A (EC 3.4.17.1)

The carboxypeptidase A (CPA) used in these metabolism studies was a commercially available preparation (Sigma Chemicals) purified from bovine pancreas. The enzyme was suspended in an aqueous solution containing toluene and had been treated with phenylmethylsulphonylfluoride (PMSF) to inhibit serine proteases. Each mg of protein was equivalent to 70.5 units, where each unit was capable of hydrolysing 1.0 µmole of hippuryl-L-phenylalanine per min at pH 7.5 and at a temperature of 25°C. The enzyme preparation contained 16.2 mg/mL of protein. The activity per mL was therefore 1.14 mmole min^{-1} under these experimental conditions.

Leucine aminopeptidase (cytosolic) (EC 3.4.11.1)

The leucine aminopeptidase (cytosolic) enzyme (LAP-C) was a commercially available preparation (Sigma Chemicals), obtained from porcine kidney. The enzyme was a chromatographically purified suspension in 2.9M $(NH_4)_2SO_4$, 0.1M Tris buffer and 5 mM $MgCl_2$ solution at pH 8.0. Each mg of protein was equivalent to 210 units, where each unit was capable of hydrolysing 1.0 µmole of L-leucinamide to L-leucine and NH_3 per min at pH 8.5 and at a temperature of 25°C. The enzyme preparation contained 1.9 mg mL-1 of protein. The activity per mL was therefore 0.399 mmole min^{-1} under these experimental conditions.

Leucine aminopeptidase (microsomal) (EC 3.4.11.2)

The leucine aminopeptidase (microsomal) enzyme (LAP-M) was a commercially available preparation (Sigma Chemicals), obtained from porcine kidney microsomes. The enzyme was a suspension in 3.5M $(NH_4)_2SO_4$ and 10 mM Mg Cl_2 solution at pH 7.7. Each mg of protein was equivalent to 19 units, where each unit was capable of hydrolysing 1.0 µmole of L-leucine-p-nitroanilide to L-leucine and p-nitroaniline per min at pH 7.2 and at a temperature of 37°C. The enzyme preparation contained 2.1 mg mL^{-1} of protein. The activity per mL was therefore 39.9 mole min^{-1} under these experimental conditions.

7.3.1.4 Preparation of nasal wash

The wash was carried out according to the method of Hussain et al. (1990) employing sheep rather than healthy, live human beings. The heads of three recently slaughtered sheep were obtained. Each half of the nasal cavity was washed with five, 1 mL portions of 0.1M phosphate buffer (pH 7.4), and the washings collected and mixed. These washings were stored on ice whilst the nasal mucosal homogenate was prepared. The wash preparation was equilibrated for 30 min at 37°C immediately prior to use.

The nasal homogenate was prepared as described in section 7.3.1.1. After the protein concentration had been determined by the method of Bradford (1976) as described in section 3.3.3.2, the homogenate was diluted to the same protein concentration as the wash (0.63 mg mL^{-1}) with phosphate buffer.

7.3.2 **Metabolism studies**

All the metabolism studies were carried out in duplicate, in a shaking water bath at 37°C. The concentrations of substrates and metabolites were determined by HPLC as described in section 2.3.2.

7.3.2.1 To determine the effect of storage conditions on the enzymic activity of sheep nasal mucosa

The substrates employed to test the storage conditions of the tissue upon the the proteolytic activity were the tripeptides trp-gly-gly (WGG) and phe-gly-gly (FGG). Stock solutions (3 mM) or (30 mM) of the substrates were prepared in 0.1M phosphate buffer (pH 7.4).

Fresh nasal mucosa was pooled and divided into 1 g portions. Some of the tissue was then homogenised as described in section 7.3.1.1. Sufficient of the homogenate to carry out the metabolism of WGG and FGG with fresh homogenate was retained, while the remainder of the homogenate, and the unprocessed nasal mucosal tissue was stored at -70°C, in 0.5 mL and 1 g samples respectively.

After one and three week periods, the stored tissue was defrosted at room temperature for 30 min and an homogenate prepared. The stored, frozen homogenate was similarly defrosted at room temperature. These two enzymic preparations were then used in metabolism studies.

The following protocol was adopted for all the experiments. The enzymic preparation (0.1 mL) was diluted in 3.9 mL phosphate buffer (pH 7.4) and the protein concentration determined. The metabolism was initiated by the addition of 2 mL of the substrate (final concentration 1 mM or 10 mM). At set time points 0.4 mL samples were removed from the incubation mixture and the reaction quenched on ice by the addition of 0.1 mL of trifluoroacetic acid (TFA). The sample was then centrifuged for 10 min at 9000 *g* to precipitate the protein.

Samples (100 µL), of each time point sample (comprising 0.4 mL peptide mixture in 0.1M phosphate buffer (pH 7.4), and 0.1 mL TFA), were injected onto the HPLC column without any sample pretreatment. HPLC standards were prepared in 0.1M phosphate buffer (pH 7.4). Samples (100 µL) of TFA were then added to 0.4 mL samples of each standard and 100 µL of the resultant mixture injected onto the analytical column.

7.3.2.2 Homogenate preparation

The substrates tested comprised a tetrapeptide phenylalanylglycyl-glycylphenylalanine (FGGF), tripeptides phenylalanylglycylglycine (FGG), glycylglycylphenylalanine (GGF), and tryptophylglycylglycine (WGG) and dipeptides phenylalanylglycine (FG), glycylphenylalanine (GF) and tryptophylglycine (WG). In preliminary experiments to validate the method 3 mM stock solutions of the substrates were prepared in 0.1M phosphate buffer (pH 7.4). Subsequently, due to the insolubility of FGGF, 3 mM stock solutions of all the substrates were prepared in 20:80 dimethylsulphoxide:phosphate buffer to enable direct comparisons to be made.

The homogenate (0.1 mL) was diluted in 3.9 mL phosphate buffer and the protein concentration determined. The metabolism was initiated by the addition of 2 mL of the substrate (final concentration 1 mM). At set

time points (0 - 420 min) 0.4 mL samples were removed from the incubation mixture and the reaction quenched as described in section 7.3.2.1.

Samples (100 µL), of each time point sample (comprising 0.4 mL peptide mixture in 6.67% DMSO in 0.1M phosphate buffer (pH 7.4), and 0.1 mL TFA), were injected onto the HPLC column without any sample pretreatment. HPLC standards were prepared in 6.67% DMSO in 0.1M phosphate buffer (pH 7.4). Samples (100 µL) of TFA were then added to 0.4 mL samples of each standard and 100 µL of the resultant mixture injected onto the analytical column

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7.3.2.3 Effect of pH upon the rate of hydrolysis

To determine the effect of pH upon the reaction the substrate GGF was prepared in McIlvaine's buffers of pH's 3.4, 5.4 and 7.4 at a 3 mM concentration. The buffers were prepared to the same ionic strength with KCl (Appendix 2).

The same protocol as 7.3.2.2 was then followed but substituting McIlvaine's buffer for phosphate and excluding DMSO. The HPLC procedures were as described in section 7.3.2.1 but using McIlvaine's buffer.

7.3.2.4 Experiments performed with enzymic fractions

The substrate GGF was prepared in McIlvaine's buffer (pH 7.4).

Each of the three enzymic preparations (0.1 mL) were diluted in 3.9 mL McIlvaine's buffer (pH 7.4) and the protein concentration of the resultant solutions determined. The metabolism was initiated by the addition of 2 mL of the substrate (final concentration 1 mM). At set time points (0 - 300 min) 0.4 mL samples were removed from the incubation mixture and the reaction quenched as described in section 7.3.2.1.

The HPLC procedures were as described in section 7.3.2.1 but using McIlvaine's buffer.

7.3.2.5 Pure enzymes

The substrates tested comprised the three tripeptides FGG, GGF and WGG and the dipeptide FG. The substrates (3 mM) were prepared in 0.1M phosphate buffer

The metabolism was initiated by the addition of 2 mL of the appropriate substrate (final concentration 1 mM) to the diluted enzymic preparation. At set time points (0 - 420 min) 0.4 mL samples were removed from the incubation mixture and the reaction quenched as described in section 7.3.2.1.

The HPLC procedures were as described in section 7.3.2.1 but using McIlvaine's buffer.

To determine whether the activity of carboxypeptidase was inhibited by citric acid or potassium chloride

The substrate GGF (3 mM) was prepared in one of three solutions. Solution A comprised of 90.6 mL of 0.2M Na_2HPO_4, diluted to 100 mL with water and adjusted to pH 7.4 with *o*-phosphoric acid. Solution B comprised phosphate buffer and citric acid (pH 7.4) after McIlvaine. Solution C contained the same reagents as B, with additional 0.298 g KCl.

The same protocol was used as in section 7.3.2.5

7.3.2.6 Wash preparation

The metabolism studies were carried out according to the method of Hussain et al. (1990). The substrates tested were the pentapeptide leucine enkephalin (YGGFL) and the tetrapeptide des-tyrosine leucine enkephalin (GGFL). Stock solutions of YGGFL and GGFL (120 µg mL^{-1}) were prepared in 0.1M phosphate buffer (pH 7.4)

Samples (0.5 mL) of the nasal wash, nasal mucosal homogenate and phosphate buffer were equilibrated at 37°C in a water bath for 30 min. The reaction was initiated by the addition of 100 µL of the YGGFL or GGFL solution to the enzymic preparations or the buffer, to give a

final enkephalin concentration of 20 μg mL^{-1}. At set time points 100 μL samples were removed from the incubation mixture and the reaction quenched, on ice, with 50 μL 0.2M citrate buffer (pH 2.3).

Samples (20 μL), taken from each time point sample (comprising 100 μL peptide mixture in 0.1M phosphate buffer (pH 7.4), and 50 μL of citrate buffer), were injected onto the HPLC column without any sample pretreatment. HPLC standards were prepared in 0.1M phosphate buffer (pH 7.4). Samples (50 μL) of citrate buffer were then added to 100 μL samples of each standard and 20 μL of the resultant mixture injected onto the analytical column.

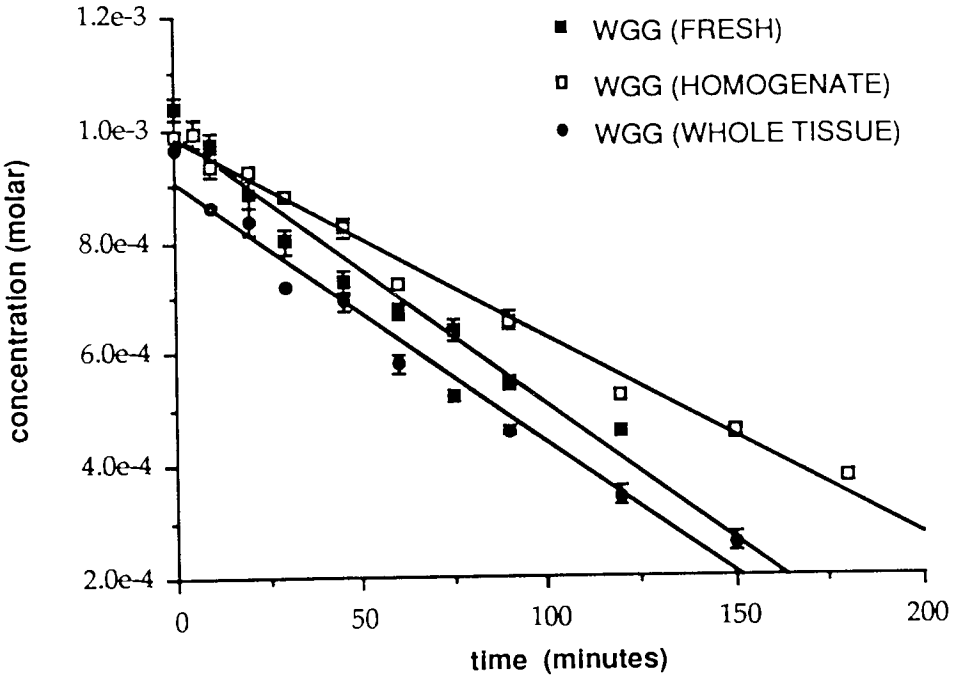
7.4 RESULTS AND DISCUSSION

7.4.1 Effect of storage conditions on the enzymic activity of sheep nasal mucosa.

The effect of freezing on the activity of sheep nasal mucosa was investigated with the substrates WGG and FGG. The results shown in figures 7.2 and 7.3 demonstrated that storage of the nasal mucosa in an unprocessed state indicated by WHOLE TISSUE on the graph, did not significantly reduce the proteolytic activity. The rate of degradation of WGG (FRESH) was 4.87 x 10^{-6} mol L^{-1} min^{-1} and WGG (WHOLE TISSUE) 4.66 x 10^{-6} mol L^{-1} min^{-1}. A similar result was obtained with 1mM FGG where the rates of degradation of FGG were (FRESH) 2.02 x 10^{-5} mol L^{-1} min^{-1} and (WHOLE TISSUE) 1.90 x 10^{-5} mol L^{-1} min^{-1}.

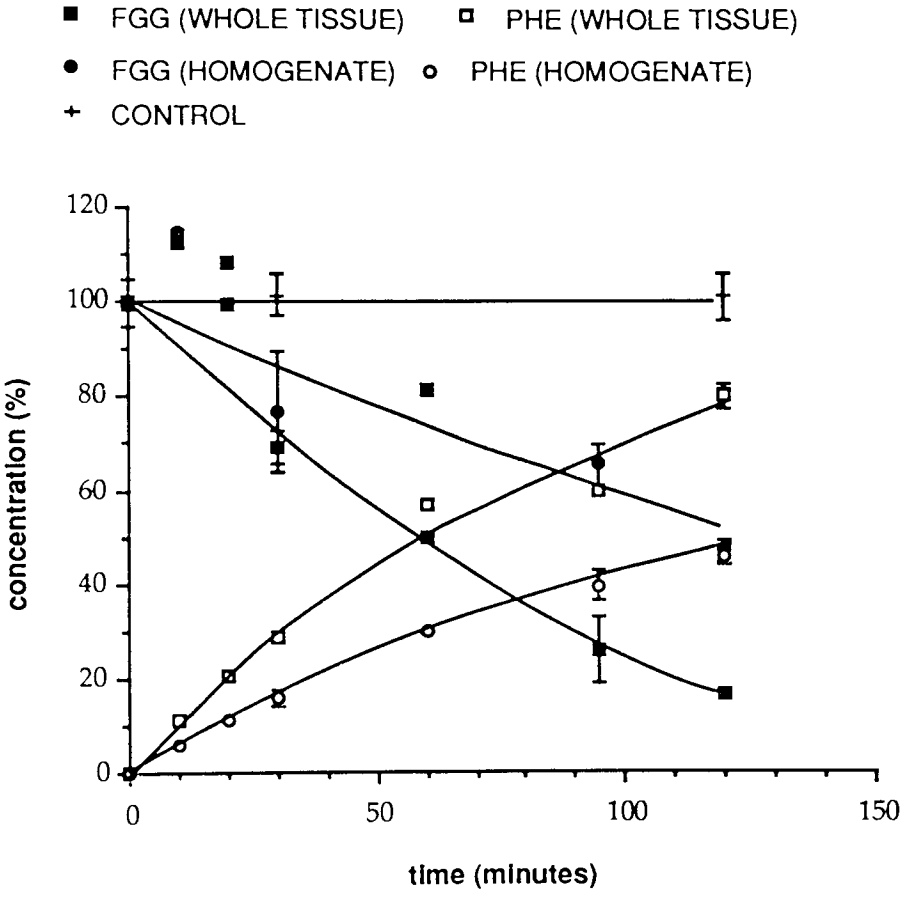
Homogenisation of the nasal mucosa and then storage of the resultant homogenate preparation at -70°C however, caused a reduction in enzymic capability. In the case of WGG the rate of degradation with the stored homogenate was 80% of the value obtained with the fresh mucosa. The results with 10 mM FGG showed a marked difference in the rates of degradation of the stored homogenate (5.72 x 10^{-5} mol L^{-1}

Figure 7.2 To demonstrate the effect of storage conditions on the metabolizing capability of sheep nasal mucosa expressed against tryptophylglycylglycine (WGG). Where (FRESH) indicates that the homogenate was prepared from unstored nasal mucosa, (HOMOGENATE) represents the tissue that was stored as an homogenate and (WHOLE TISSUE) indicates the tissue was stored in an unprocessed form. Points are the mean ± sem of two determinations.



tissue storage condition	intercept ($\times 10^4$)	slope ($\times 10^6$)	r
FRESH	9.96	4.87	0.984
HOMOGENATE	9.82	3.54	0.994
WHOLE TISSUE	9.05	4.66	0.986

Figure 7.3 To demonstrate the effect of storage conditions on the metabolizing capability of sheep nasal mucosa expressed against phenylalanylglycylglycine (FGG). Where (FRESH) indicates that the homogenate was prepared from unstored nasal mucosa, (HOMOGENATE) represents the tissue that was stored as an homogenate and (WHOLE TISSUE) indicates the tissue was stored in an unprocessed form. Points are the mean ± sem of two determinations.



min^{-1}) and the stored unprocessed mucosa (8.79×10^{-5} mol L^{-1} min^{-1}). These results are similar to those observed by Stratford and Lee (1986) with rabbit nasal mucosa. In the light of this result, tissue was stored in an unprocessed form and defrosted immediately prior to use in metabolism studies.

7.4.2 Metabolism studies with homogenate preparation

Control experiments demonstrated that all of the peptides studied were chemically stable in 0.1M phosphate buffer with 6.67% DMSO, at 37°C, for the duration of the experiments. The control homogenate experiments showed that the preparation did not interfere with the HPLC assay. In preliminary experiments, the enzymic activity against WGG, FGG and GGF, dissolved in phosphate buffer containing 6.67% DMSO, was shown not to be markedly different to the activity in phosphate buffer alone (data not shown). In contrast, all of the substrates tested were hydrolysed in the nasal mucosal homogenate but with a considerable variation in susceptibility to degradation. The protein concentration of the homogenate was determined to be 0.42 mg mL^{-1} in the incubation mixture.

The degradation profiles of all the substrates tested is shown in figure 7.4. It can be seen that the dipeptide GF was hydrolysed most rapidly and was undetectable after 10 min. In comparison WGG, the most stable peptide, was still quantifiable after 420 min. The three tripeptides showed considerable variation in stability, with the order of stability being FGG < GGF < WGG with half-lives of 8, 41 and 95 min respectively, as determined by semilogarithmic plots of time versus concentration. The order of stability for the three dipeptides was GF < FG < WG. The tetrapeptide FGGF was of similar stability to GGF with a half-life of 44 min. The first-order kinetic parameters are presented in table 7.2. In the case of the dipeptide FG the semilogarithmic plot of time versus concentration was not linear and therefore the first-order kinetic parameters were not quantifiable.

Figure 7.4 The degradation of phenylalanylglycylglycylphenylalanine (FGGF), phenylalanylglycylglycine (FGG), phenylalanylglycine (FG), glycylglycylphenylalanine (GGF), glycylphenylalanine (GF), tryptophylglycylglycine (WGG) and tryptophylglycine (WG) by nasal mucosal homogenate in 0.1M phosphate buffer containing 6.67% DMSO (pH 7.4) at 37°C. Points are the mean ± sem of two determinations.

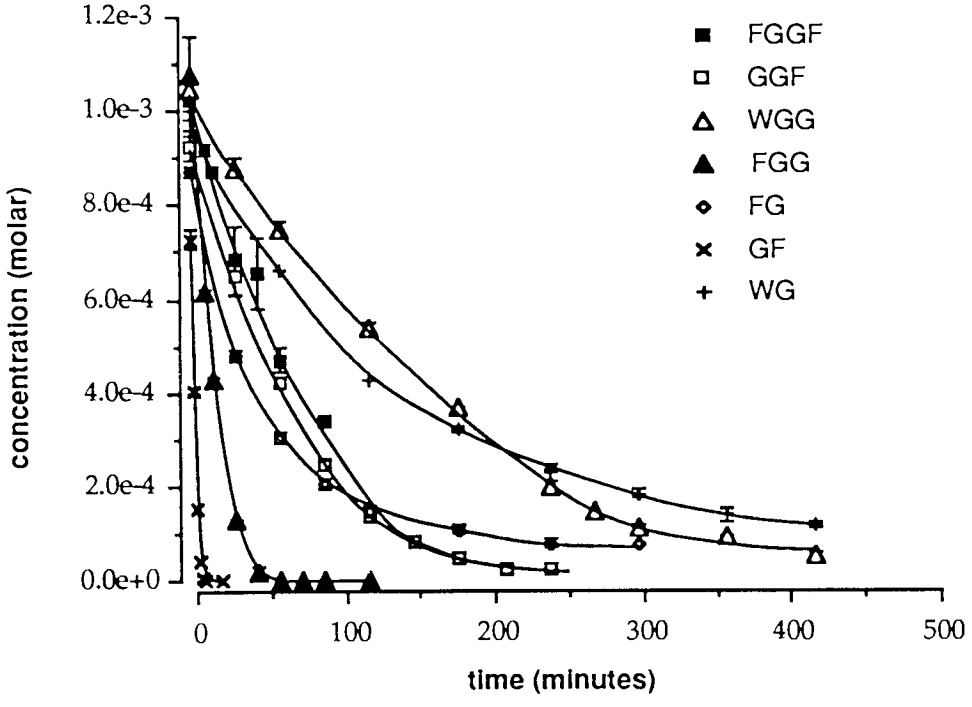


Table 7.2 First-order kinetic parameters determined for substrates in homogenate preparation.

Substrate	half-life (min)	k (10^{-s} min^{-1})
FGGF	44	15.84
FGG	8	88.75
WGG	95	7.31
GGF	41	16.85
GF	1	594.7
WG	136	5.08

Some trends are apparent but it is difficult to generalise on structure-activity relationships as only seven compounds were evaluated. For example both compounds with aliphatic N-terminal amino acids, GF and GGF, were degraded rapidly. GF was least stable with a half-life of 2 minutes and GGF was the third least stable with a half-life of 41 minutes. A similar trend was observed by Sterchi and Woodley (1980), who studied the metabolism of a series of peptides, (including FG, FGG and FGGF) with homogenates of human small intestinal mucosa and concluded that specific activities were significantly higher towards peptides with aliphatic, rather then aromatic amino acids at their N-terminal end. In contrast, is the observation that FGG was degraded considerably faster than GGF in the sheep nasal homogenate.

Bachmair et al. (1986) have also suggested that the intracellular half-life of a protein may be a function of its amino terminal residue. Other possible correlations, are that short peptide sequences in a protein determine the rate of degradation (Dice et al., 1986; Rogers et al., 1986)

The Michaelis-Menten kinetic parameters associated with these reactions were determined using the half-time analysis method described in section 7.2. Theoretical hydrolysis profiles generated with these parameters, as described in section 7.2, were found to be

in close agreement with the experimental profiles, with the exception of WG. A summary of the results is given in table 7.3.

Table 7.3 Michaelis-Menten kinetic parameters determined by the method of half-time analysis for substrates in homogenate preparation. Where 1 represents the **most labile** compound and 5 the **most** stable.

Substrate	V_{max} (10-5 mol L-1 min-1)	K_m (10-4 M)	lability
FGGF	1.90	8.23	4
FGG	5.43	4.45	2
WGG	0.54	3.48	5
GGF	2.69	12.36	3
GF	33.25	4.25	1

The kinetic parameter V_{max}, indicates the maximum rate of degradation obtainable and K_m is often described as the affinity of an enzyme for a particular substrate. Hence, a high V_{max} and low K_m would indicate that the compound was a good substrate, displaying high affinity for the enzyme. The compound would therefore be rapidly degraded. In contrast, a low V_{max} and high K_m (low affinity) would imply the compound was a poor substrate. The substrate GF, which was hydrolysed most rapidly, exhibited a low K_m value (4.25×10^{-4} M) and a large V_{max} value (33.25×10^{-5} mol min-1) indicating that it was a good substrate. Conversely, the most stable compound WGG, had a small V_{max} value (0.34×10^{-5} mol min-1) and a high K_m (10.08×10^{-4}M), confirming that the compound was a poor substrate.

The data obtained from the hydrolysis of FG did not produce linear plots from half-time analysis and consequently Michaelis-Menten kinetic parameters for this substrate were not determined. Computer modelling introducing enzyme inhibition produced similar non-linear results (Irwin, 1991). The presence of enzyme inhibition in the hydrolysis of FG was not determined experimentally.

While the advantage of an homogenate preparation is that it gives an indication of the total enzymic activity of a tissue, the preparation presumably contains several different enzymes, any of which might be capable of degrading a given substrate. The result is that with such a preparation, it is difficult to draw conclusions on the specificities of the enzymes, as different enzymes may be catalysing different reactions.

The HPLC analysis enabled the degradation pathway to be elucidated in addition to providing kinetic information for the degradation profile. The degradation of the tetrapeptide FGGF is illustrated in figure 7.5. FGGF was cleaved exclusively at its N-terminal end, as indicated by the absence of the possible metabolites FGG, FG and GF, to generate the amino acid phenylalanine and the tripeptide GGF. The GGF formed was then cleaved, exclusively at its C-terminal end, to yield phenylalanine, as denoted by the absence of GF. This suggested that the dipeptide product glycylglycine (GG) was the other product of the hydrolysis of GGF, but this was not confirmed experimentally.

The half-time analysis of FGGF, determined from the degradation profile illustrated in figure 7.5, is illustrated in figure 7.6, as an example of plots produced by this methodology. For FGGF the equation of the line was determined by linear regression as:

$$y = 30.01 \pm 2.67 \times 10^{4}.So \qquad (7.8)$$

with a correlation coefficient of 0.993. The parameters V_{max} and K_m for FGGF and the other substrates tested are presented in table 7.3.

Figure 7.5 The hydrolysis profile of the tetrapeptide phenylalanyl-glycylglycylphenylalanine (FGGF) by nasal mucosal homogenate in 0.1M phosphate buffer containing 6.67% dimethylsulphoxide at 37°C. Points are the mean ± sem of two determinations.

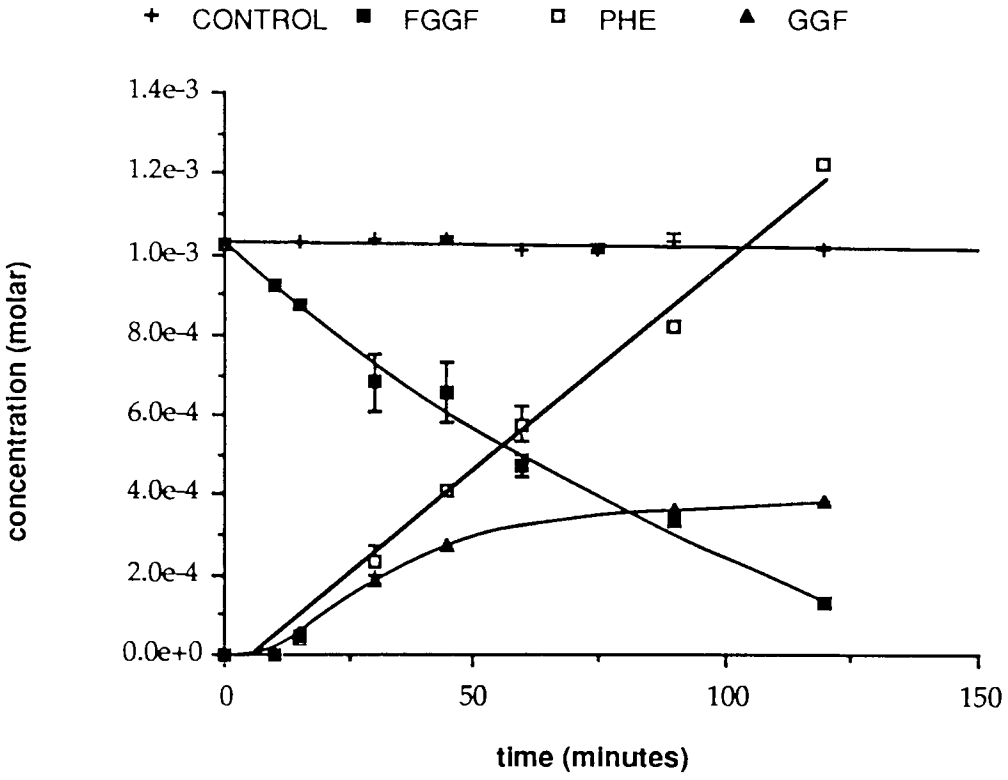
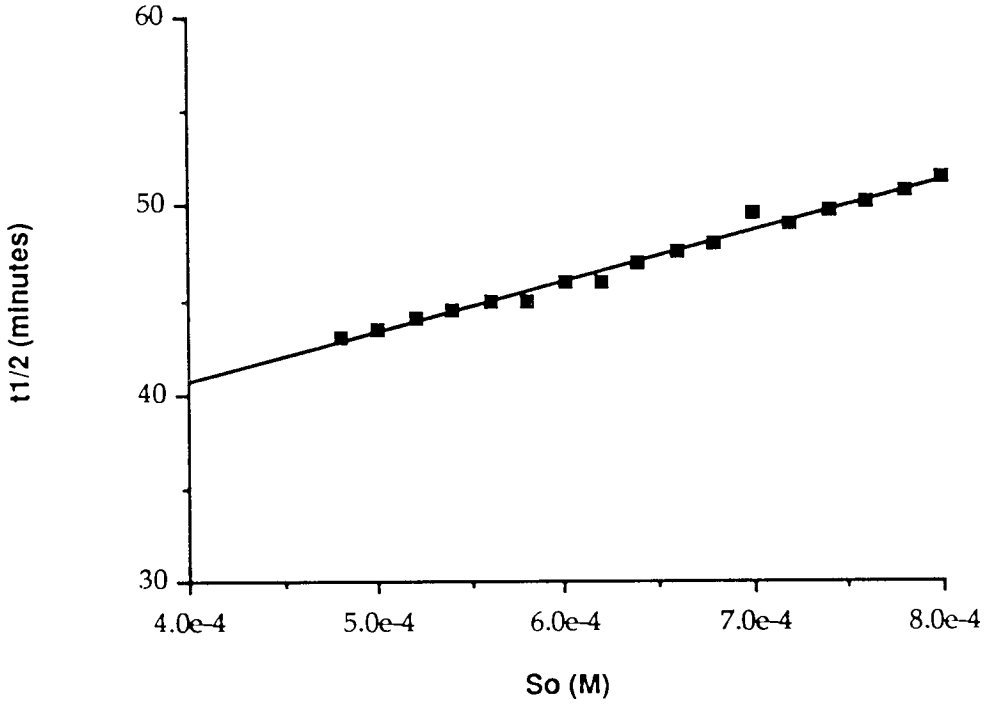
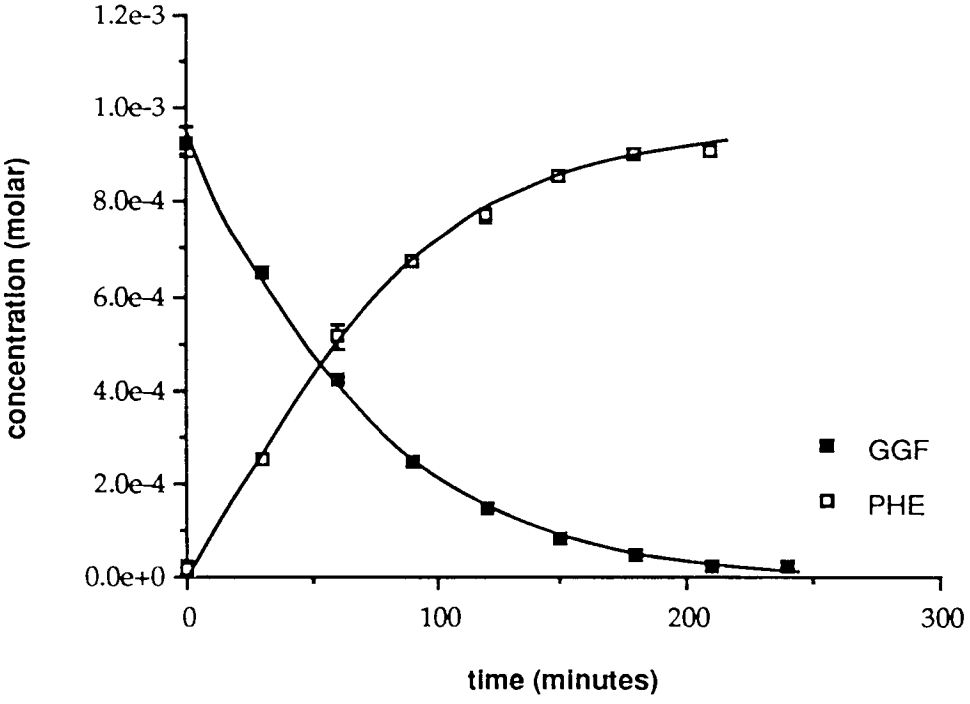


Figure 7.6 Half-time analysis of the degradation profile of phenylalanylglycylglycylphenylalanine.



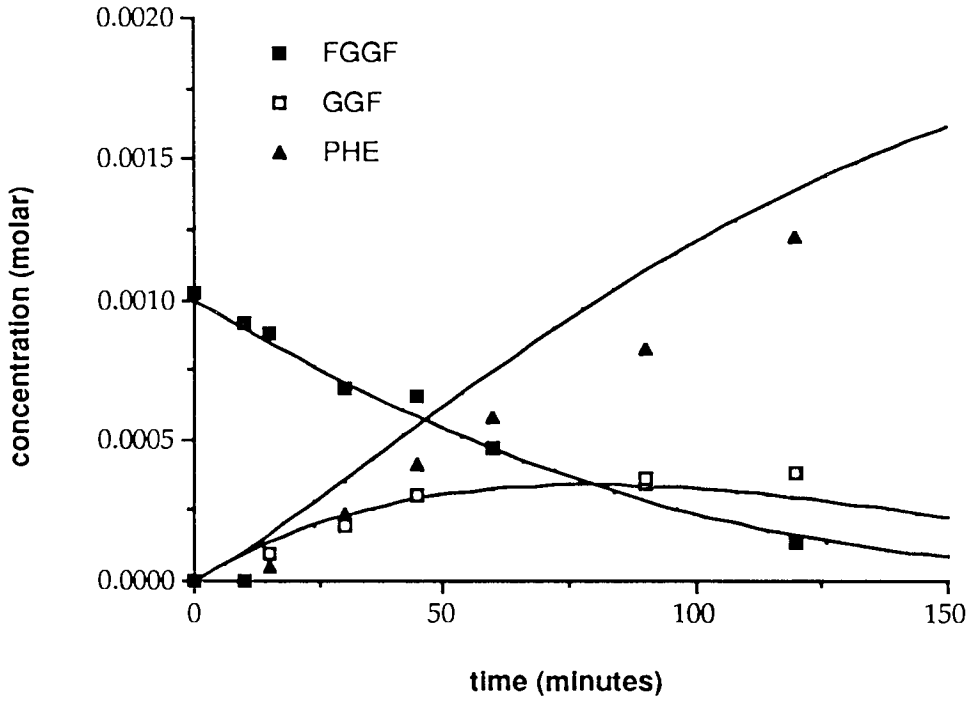
The tripeptide GGF, in contrast to FGGF, was degraded exclusively at its C-terminal end to yield the product phenylalanine. The product GF was not observed. This is illustrated in figure 7.7.

Figure 7.7 The hydrolysis profile of glycylglycylphenylalanine (GGF) to phenylalanine (PHE) by nasal mucosal homogenate in 0.1M phosphate buffer containing 6.67% dimethylsulphoxide at 37°C. Points are the mean ± sem of two determinations.



Using the kinetic parameters K_m and V_{max} determined for FGGF and GGF from figures 7.6 and 7.7, it was possible to construct expected profiles for FGGF and GGF from sequential analysis of the Michaelis-Menten equation in order to confirm the proposed route of degradation. The expected profiles for the degradation of FGGF, and formation of its metabolites are presented in figures 7.8.

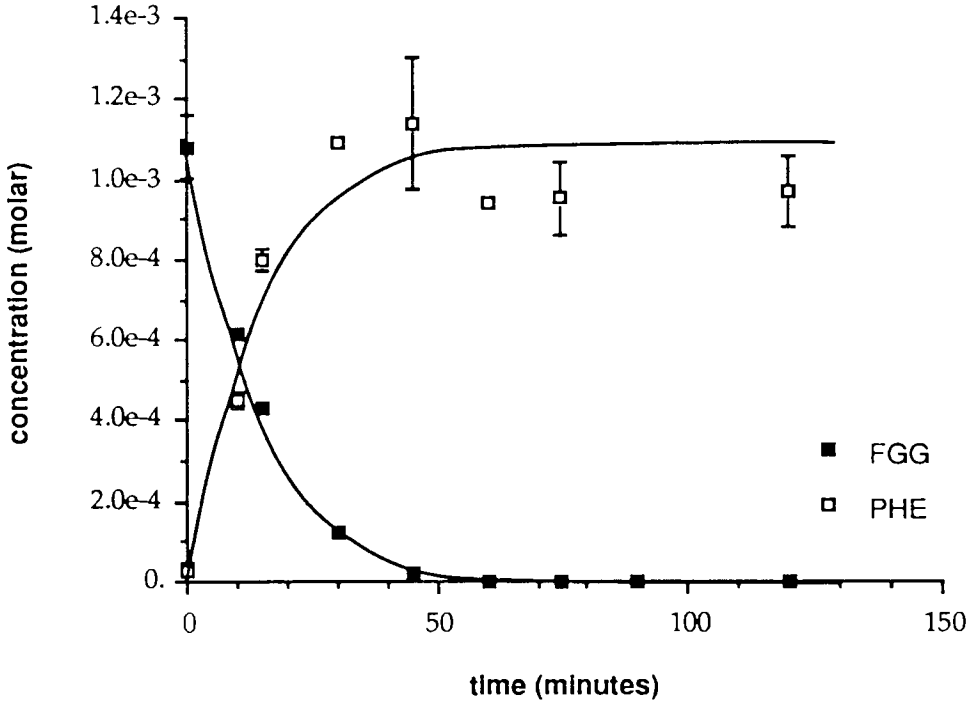
Figure 7.8 Comparison of the experimental and theoretical hydrolysis profiles of phenylalanylglycylglycylphenylalanine (FGGF). The symbols represent the mean ± sem of two determinations. The lines represent theoretical values generated by sequential computer analysis of the Michaelis-Menten equation using the experimentally determined Michaelis-Menten kinetic parameters for FGGF and GGF.



The theoretical progress curves for FGGF and GGF closely mirrored the experimental data for degradation of the substrate and formation of products, presented in figures 7.5 and 7.7. This suggested that the proposed routes of degradation for these compounds were valid, as were the determined kinetic parameters. Theoretical progress curves were also constructed by iteration using the computer program NONREG and the experimentally determined first-order rate constants for the degradation of FGGF and GGF. These theoretical progress curves also satisfactorily described the experimental profiles (data not shown).

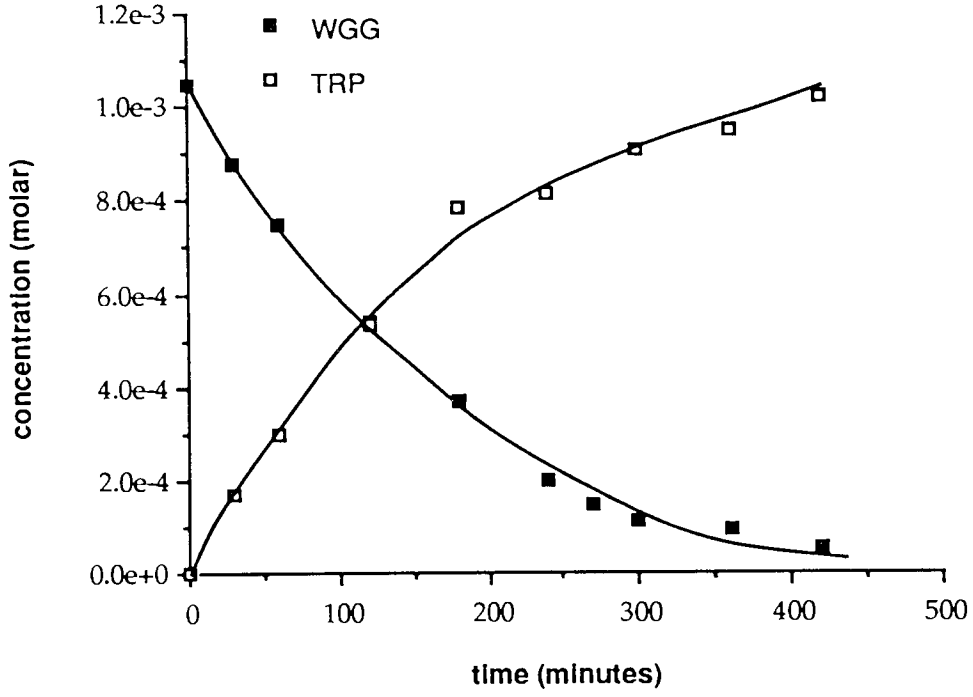
The hydrolysis of the tripeptide FGG, like the tetrapeptide FGGF, occurred exclusively at the N-terminal to generate phenylalanine, whilst no FG was observed, which would be present as a result of carboxypeptidase action on this compound. This is illustrated in figure 7.9. This route was confirmed by sequential analysis of the Michaelis-Menten equation (data not shown).

Figure 7.9 The hydrolysis profile of phenylalanylglycylglycine (FGG) to phenylalanine (PHE) by nasal mucosal homogenate in 0.1M phosphate buffer containing 6.67% dimethylsulphoxide at 37°C. Points are the mean ± sem of two determinations.



Similarly, the tripeptide WGG was also hydrolysed exclusively at its N-terminal end to generate tryptophan and the dipeptide GG, with no indication of the metabolite WG. This is illustrated in figure 7.10. The degradation route was confirmed by sequential analysis of the Michaelis-Menten equation (data not shown).

Figure 7.10 The hydrolysis profile of tryptophylglycylglycine (WGG) by nasal mucosal homogenate to tryptophan (TRP) in 0.1M phosphate buffer containing 6.67% dimethylsulphoxide at 37°C. Points are the mean ± sem of two determinations.



Each of the dipeptides FG, GF and WG was converted to its constituent amino acids PHE, PHE and TRP respectively. The amino acid glycine was not quantified, but would be expected to be formed from the mass balance between each of the dipeptide and the formation of the constituent aromatic amino acid. The plots for FG, GF and WG are illustrated in figures 7.11, 7.12 and 7.13 respectively.

Figure 7.11 The metabolism of phenylalanylglycine (FG) to phenylalanine (PHE) by nasal mucosal homogenate in 0.1M phosphate buffer containing 6.67% dimethylsulphoxide at 37°C. Points are the mean ± sem of two determinations.

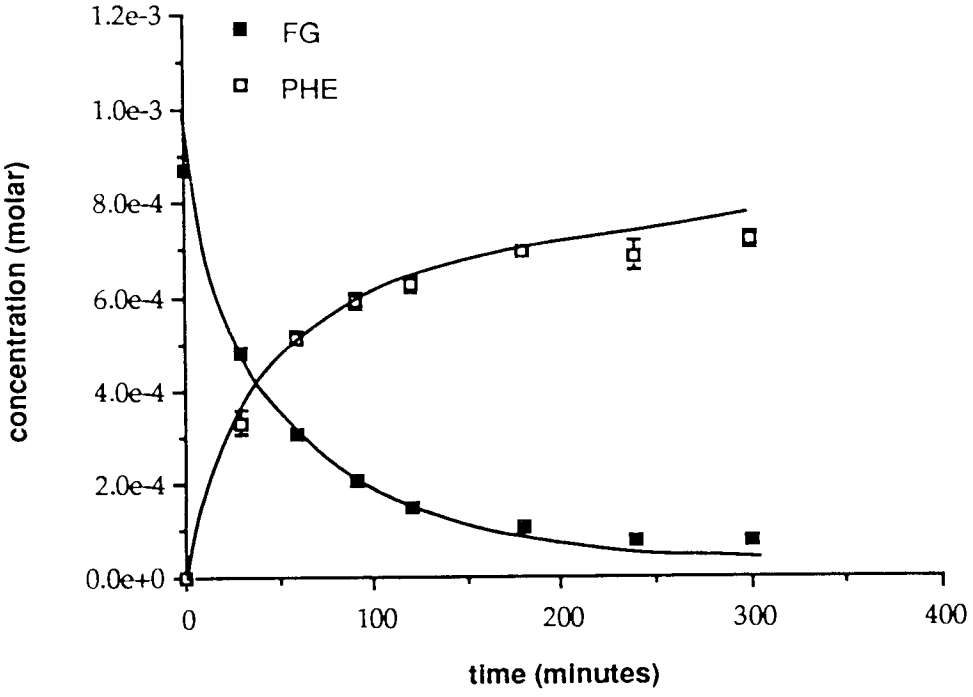


Figure 7.12 The metabolism of glycylphenylalanine (GF) to phenylalanine (PHE) by nasal mucosal homogenate in 0.1M phosphate buffer containing 6.67% dimethylsulphoxide at 37°C. Points are the mean ± sem of two determinations.

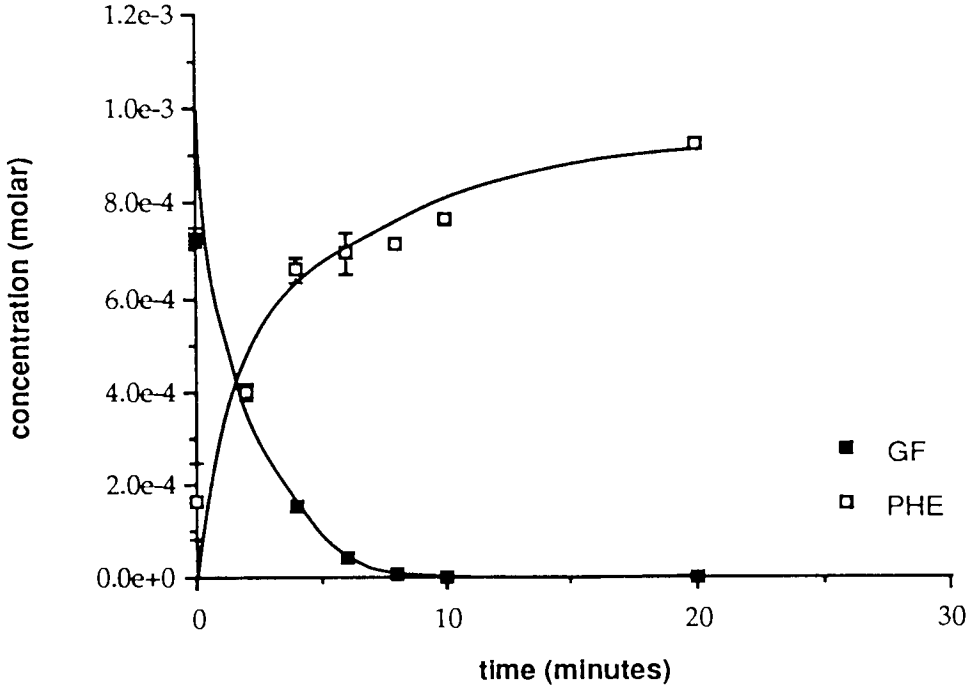
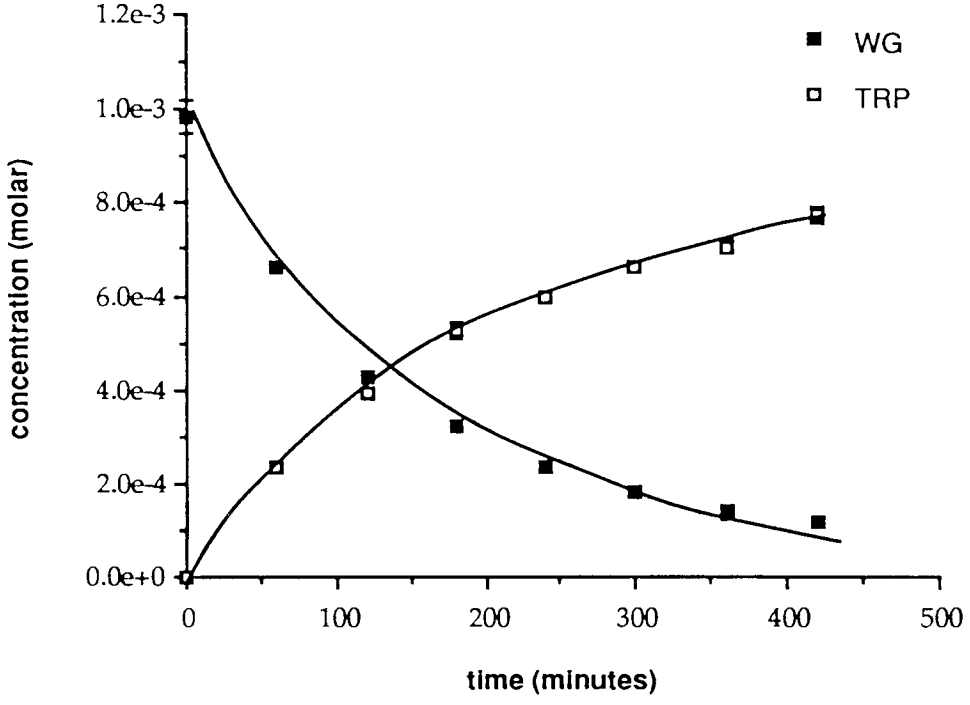
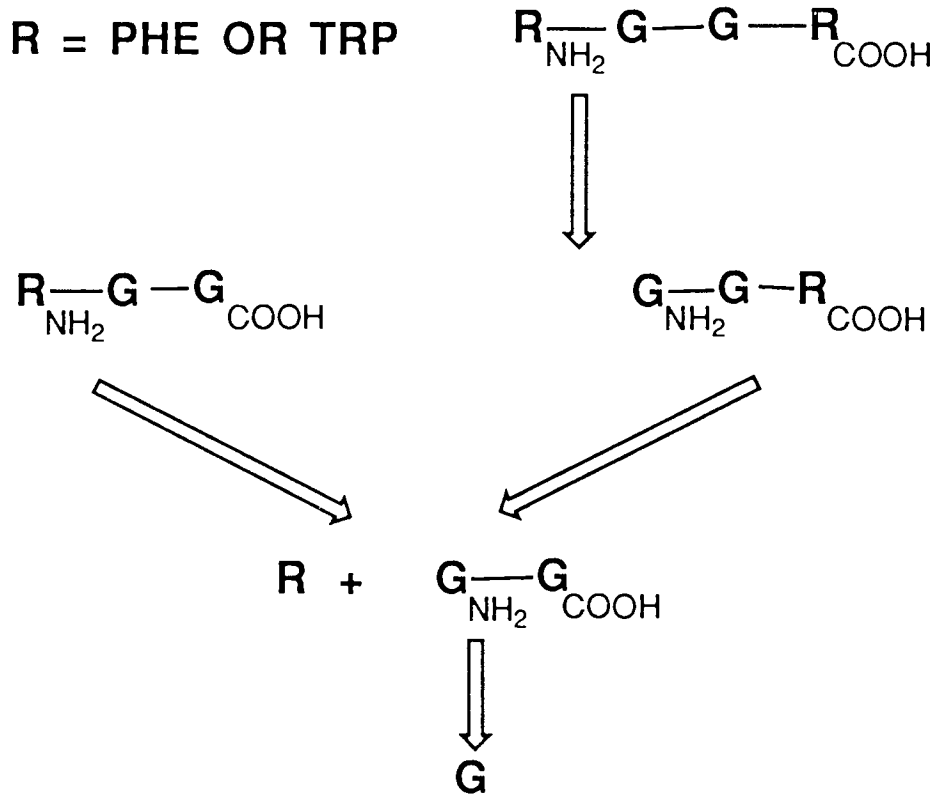


Figure 7.13 The metabolism of tryptophylglycine (WG) to tryptophan (TRP) by nasal mucosal homogenate in 0.1M phosphate buffer containing 6.67% dimethylsulphoxide at 37°C. Points are the mean ± sem of two determinations.



The tetrapeptide, FGGF, and the three tripeptides, FGG, GGF and FGG, were each cleaved exclusively at the peptide bond between the aromatic amino acid and the glycine irrespective of whether the amino acid was at the N-terminal or the C-terminal end of the peptide. This observation suggests some specificity for aromatic amino acids may exist. This is illustrated schematically in figure 7.14.

Figure 7.14 A schematic representation summarising the degradation pathways observed with the homogenate preparation.



The susceptibility of FGGF, WGG, and FGG to attack at their N-terminal end suggested the presence of at least one aminopeptidase. This class of proteolytic enzymes has been identified in rat and rabbit nasal mucosa (Dodda Kashi and Lee, 1986; Stratford and Lee, 1986). The degradation of GGF at its C-terminal end indicated the presence of a carboxypeptidase, which has not previously been observed in nasal tissue. The action of carboxypeptidase activity has however been observed in homogenate preparations of buccal and intestinal tissue taken from rats and hamsters (Garren and Repta, 1988). This activity was expressed as the ability of the homogenate to produce hippuric acid from hippuryl-L-phenylalanine based on the method of Folk and Schirmer (1963)

The fact that no carboxypeptidase activity was observed against FGG and WGG suggested that the enzyme may have some specificity for an aromatic, C-terminal amino acid. If this were the case, the activity may be due to carboxypeptidase A, or the lysosomal enzyme cathepsin A. Both of these enzymes exhibit a preference for either a branched or an aromatic amino acid in the C-terminal position (Iodice, 1967; Kim and Lipscomb, 1990). The action of cathepsin A may be unlikely, as lysosomal enzymes are reported to be inactivated at pH 7.4 (Bond and Beynon, 1987)

7.4.3 Effect of pH

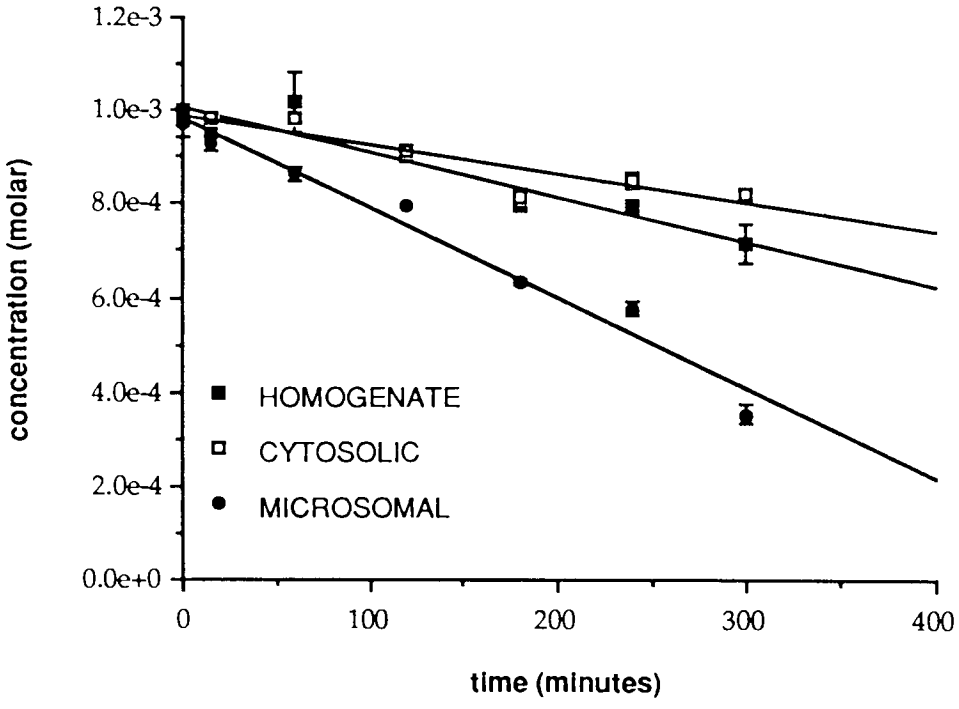
The experiments performed with the homogenate enzymic preparation were at pH 7.4, as this corresponds to the pH of the cytosol. The cellular organelle, the lysosome, however contains abundant hydrolytic enzymes which require a pH in the range 3-6.0 for optimum activity and it has been reported that at neutral pH these enzymes may be inactive (Bond and Beynon, 1987).

The effect of pH was investigated with the substrate GGF. At pH values 5.4 and 3.4 no degradation of GGF was observed (data not shown). This suggested that either GGF was stable in the presence of the lysosomal enzymes, that they had been inactivated by the homogenisation procedure or were inactive under these experimental conditions.

7.4.4 Cellular fractions

In these experiments the hydrolysis of GGF was studied using homogenate, cytosolic and microsomal fractions, prepared in McIlvaine's buffer (pH 7.4). The substrate GGF was hydrolysed in all three enzymic preparations and the degradation was found to be linear with time during the course of the experiment (300 min). This is shown in figure 7.15.

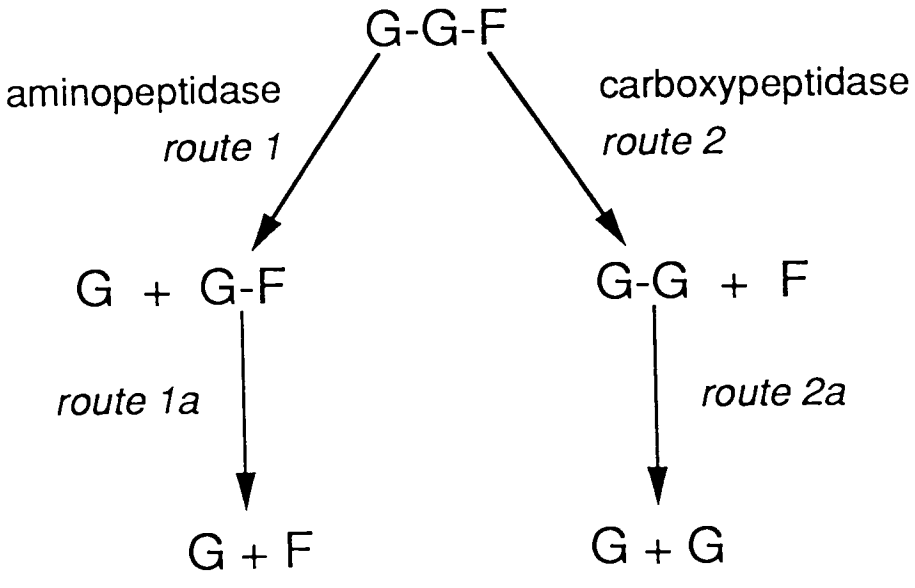
Figure 7.15 A summary of the degradation of glycylglycylphenylalanine in homogenate, cytosolic and microsomal fractions. Experiments performed in McIlvaine's buffer (pH 7.4), ionic strength 0.5M at 37°C. Points are the mean ± sem of two determinations.



The substrate was hydrolysed most rapidly in the microsomal fraction and slowest in the cytosolic fraction. The rates, determined by linear regression were 0.85 μmol L^{-1} min^{-1} (homogenate), 0.63 μmol L^{-1} min^{-1} (cytosolic) and 1.91 μmol L^{-1} min^{-1} (microsomal). The specific activities were calculated to be 0.27 μmol L^{-1} min^{-1} mg^{-1} of protein, 0.25 μmol L^{-1} min^{-1} mg^{-1} of protein and 0.54 μmol L^{-1} min^{-1} mg^{-1} of protein for the homogenate, cytosolic and microsomal fraction respectively. The activity in the microsomal fraction was therefore double that in the cytosolic fraction. This result, with greater activity being observed in membrane-bound fractions, is comparable to those obtained by Choi and Lee (1986) using methionine and leucine enkephalin with rabbit nasal mucosa and Yamamoto et al. (1988) using insulin as a substrate and rabbit nasal mucosa.

The substrate GGF may be degraded at both its N- and C-terminal ends. Attack at the N-terminal would generate the primary products GF and G, whilst attack at the C-terminal end would yield GG and F. This is illustrated in figure 7.16

Figure 7.16 A schematic diagram of the possible hydrolytic pathways for glycylglycylphenylalanine (GGF).



The experimental hydrolysis profiles for GGF in the homogenate, cytosolic and microsomal fractions are illustrated in figures 7.17, 7.18 and 7.19 respectively.

Figure 7.17 The hydrolysis profile of glycylglycylphenylalanine (GGF) in the homogenate fraction. Experiments were performed in McIlvaine's buffer (pH 7.4), ionic strength 0.5M at 37°C. Points are the mean ± sem of two determinations.

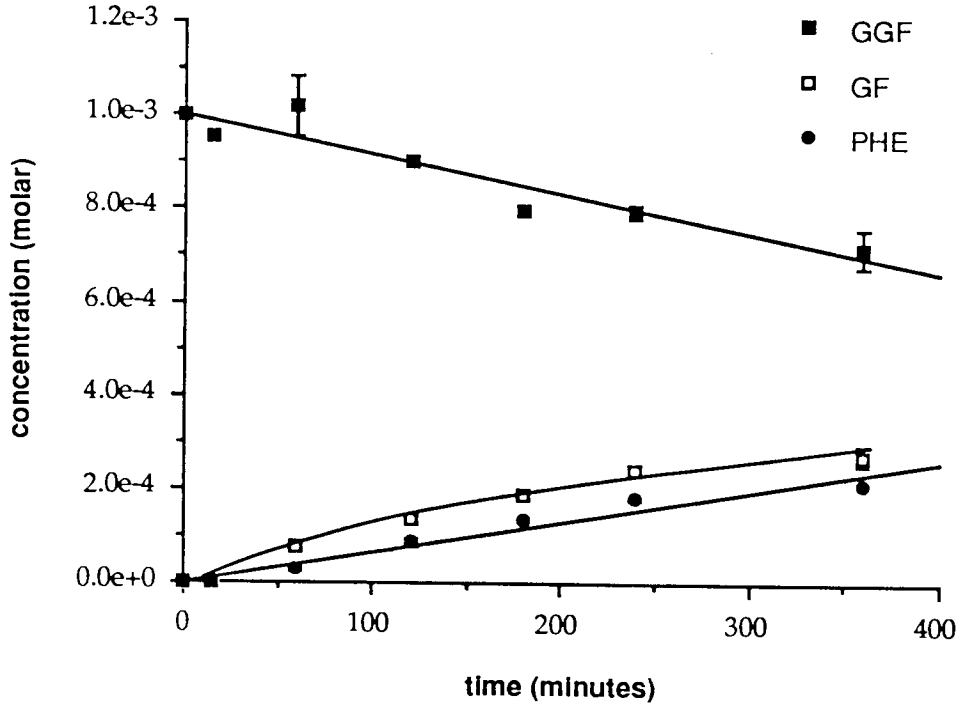


Figure 7.18 The hydrolysis profile of glycylglycylphenylalanine (GGF) in the cytosolic fraction. Experiments were performed in McIlvaine's buffer (pH 7.4), ionic strength 0.5M at 37°C. Points are the mean ± sem of two determinations.

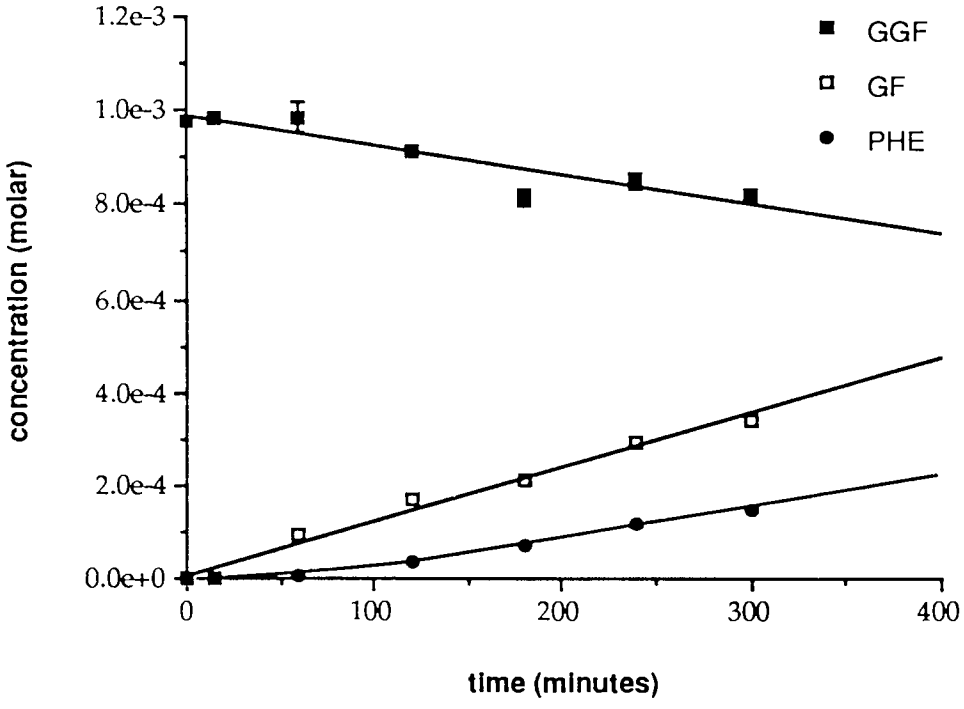
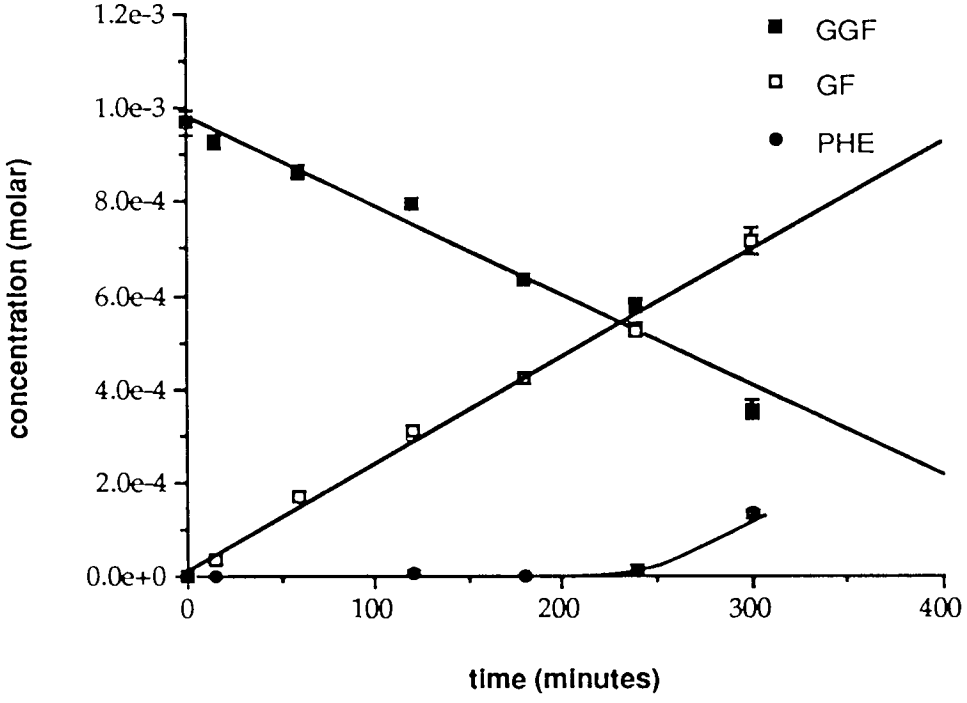


Figure 7.19 The hydrolysis profile of glycylglycylphenylalanine (GGF) in the microsomal fraction. Experiments were performed in McIlvaine's buffer (pH 7.4), ionic strength 0.5M at 37°C. Points are the mean ± sem of two determinations.



In all three preparations, the metabolites GF and PHE were observed. This was in contrast to previous experiments carried out in phosphate buffer (pH 7.4) containing 6.67% DMSO, as discussed in section 7.4.2. Under those experimental conditions GGF was degraded exclusively by carboxypeptidase action to generate phenylalanine and GG. This difference was surprising in view of the fact that the same batch of nasal mucosa was used for both sets of experiments, and control experiments showed that GGF was stable in the absence of enzymes and the enzymic mixture did not interfere with the assay.

The presence of GF and PHE indicate that degradation may have occurred at both N- and C-terminal ends of the molecule, or alternatively, that the primary products are GF and glycine from aminopeptidase action, followed by phenylalanine upon GF hydrolysis. In order to determine which routes of degradation were followed in the different enzymic fractions the ratios of GF/PHE were calculated. The ratio values are given in table 7.4.

Table 7.4 The ratios of glycylphenylalanine/phenylalanine produced in the metabolism of glycylglycylphenylalanine in each of the cellular fractions. The symbol * in the table indicates that at these time points the concentration of PHE was undetectable, consequently a ratio could not be determined.

Time (min)	homogenate	cytosolic	microsomal
0	*	*	*
15	*	*	*
60	2.90	27.11	*
120	1.55	4.62	40.93
180	1.41	2.86	28.70
240	1.43	2.56	37.97
360	1.29	2.31	5.38

The high GF/PHE ratios for the microsomal fraction suggest that attack at the N-terminal end, by aminopeptidase action is the principal site of degradation *via* route 1 and 1a in figure 7.16. The considerably lower GF/ PHE ratios in the cytosolic and homogenate fractions suggest that degradation may be occurring competitively *via* routes 1 and 2 in figure 7.16, with route 1a being very slow.

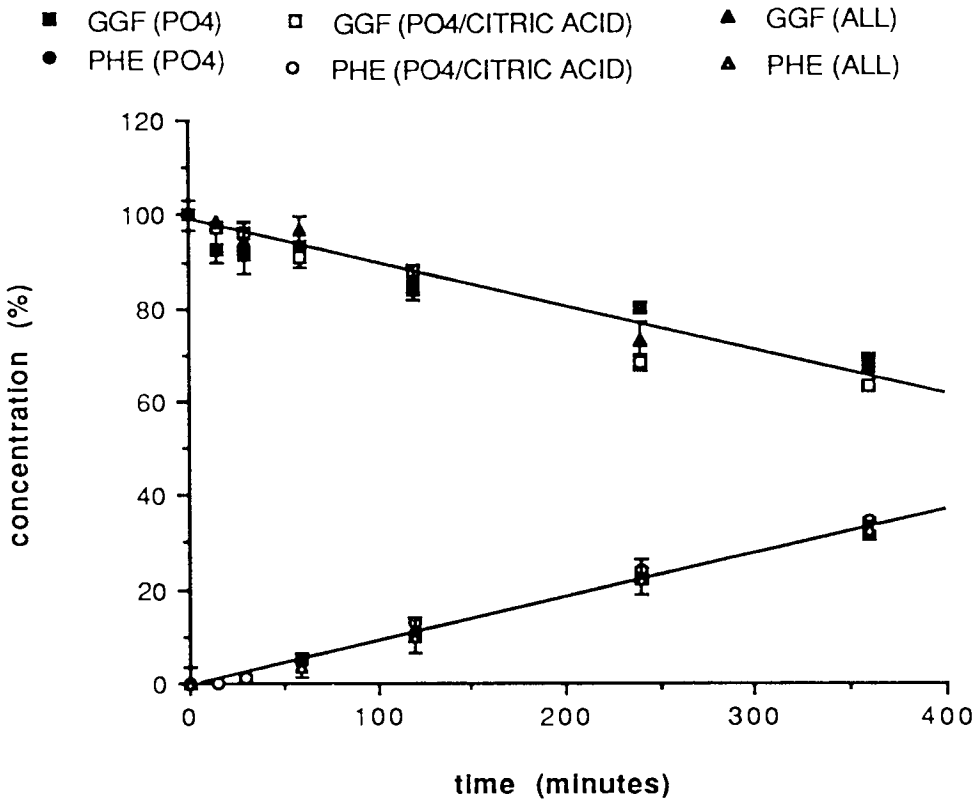
The considerable difference in rates and mechanism of GGF hydrolysis in McIlvaine's buffer compared with phosphate (section 7.4.2) may be due to a number of factors. Firstly, the enzymic activity of the nasal mucosa may have been altered during storage. The effect of storage on the activity of the tissue was assessed in section 7.4.1, with the substrates WGG and FGG, neither of which are degraded by carboxypeptidase action. A loss in carboxypeptidase activity may therefore have occurred. This experiment was not repeated with fresh nasal mucosa to confirm this result. A second possibility is that the use of McIlvaine's buffer instead of phosphate buffer may have either inhibited carboxypeptidase, or activated aminopeptidase action. McIlvaine's buffer contains phosphate and citric acid and additionally potassium chloride was added to maintain a constant ionic strength. To establish whether these extra components were responsible for the different rates and routes of degradation, experiments were performed with pure carboxypeptidase, which will be discussed in the next section.

7.4.5 Pure enzymes

The effect of citric acid and KCl on the activity of commercially available carboxypeptidase A (CPA) was assessed in an attempt to explain the difference in rates and mechanism of GGF degradation with homogenates prepared in phosphate and McIlvaine's buffer. Three different buffers A, B and C were employed, which contained phosphate buffer alone phosphate and citric acid (McIlvaine's buffer, appendix 2), or phosphate, citric acid and KCl.

As can be seen in figure 7.20 there was no difference between the degradation of GGF in the three media.

Figure 7.20 The effect of different buffers containing phosphate alone (PO_4), phosphate and citric acid (PO_4/CITRIC ACID) and phosphate, citric acid and KCl (ALL) upon the activity of carboxypeptidase A. Points are the mean ± sem of two determinations.



The rates of degradation of GGF were 0.82 μmol L^{-1} min^{-1} (buffer A) 0.12 μmol L^{-1} min^{-1} (buffer B) and 0.79 μmol L^{-1} min^{-1} (buffer C), as determined from linear regression. In each case the attack occurred exclusively at the C-terminal end to produce phenylalanine and GG apparently and no GF was observed, as would be expected for carboxypeptidase action.

The identical rates and mechanisms of degradation of GGF by pure CPA in these three buffers suggest that inhibition of carboxypeptidase is probably not the reason for the differences observed in the homogenate preparations. It is possible however that the carboxypeptidase present in the homogenate is not CPA and that it may exhibit different

activity in the two buffers employed. The effect of the different buffers on the activity of the cytosolic and microsomal LAPs was not determined. This would have indicated whether enhancement of aminopeptidase activity produced the observed results for GGF in McIlvaine's buffer.

The hydrolysis of the substrates FGG, GGF, WGG and FG was evaluated using commercially available CPA, cytosolic leucine aminopeptidase (LAP-C) and microsomal leucine aminopeptidase (LAP-M). The objective was to provide some comparison with the hydrolysis results obtained with the homogenate preparation. In each case the protein concentration of the enzyme concentrations was 10 $\mu g\ mL^{-1}$.

The relative stabilities of the different substrates are summarised in tables 7.5 and 7.6. It should be noted that only one of the substrates tested, GGF, was hydrolysed on incubation with CPA. In contrast, all of the substrates, with the exception of GGF, were hydrolysed by LAP-C but with variation in susceptibility to degradation. The order of stability was FG > FGG > WGG, with WGG being the least stable. In the studies with microsomal leucine aminopeptidase GGF surprisingly was the most rapidly degraded, but the same order of stabilities was observed for the other substrates as with the cytosolic enzyme. The rates of degradation were considerably higher with the microsomal aminopeptidase than the cytosolic aminopeptidase for all the peptides studies.

Table 7.5 The relative stabilities of each substrate in the presence of carboxypeptidase A (CPA), cytosolic leucine aminopeptidase (LAP-C) and microsomal leucine aminopeptidase (LAP-M) Where St, denotes that the substrate was stable; * that the substrate was degraded most rapidly out of the 3 enzymes; and ** that it was second most labile.

Substrate	CPA	LAP-C	LAP-M
FGG	St	**	*
WGG	St	**	*
GGF	**	St	*
FG	St	**	*

Table 7.6 The rank stabilities of the different substrates, relative to each other, in the presence of carboxypeptidase A (CPA), cytosolic leucine aminopeptidase (LAP-C) and microsomal leucine aminopeptidase (LAP-M). Where St, denotes that the substrate was stable; * that the substrate was degraded most rapidly out of the 3 enzymes; and ** that it was second most labile etc; NT that this substrate was not tested.

Substrate	CPA	LAP-C	LAP-M
FGG	St	**	***
WGG	St	*	**
GGF	*	St	*
FG	St	***	****
GF	NT	NT	*****

The first-order kinetic parameters were determined for these reactions and are tabulated in table 7.7. The rapid hydrolysis of GGF by LAP-M meant there was insufficient data to determine meaningful kinetic parameters for this substrate. This kinetic analyis was chosen due to

the successful modelling of the hydrolysis profile of FGGF (section 7.4.2) with first-order parameters and the incomplete progress curves for some of the experiments, which precluded the application of the half-time analysis methodology.

Table 7.7 First-order kinetic parameters determined from the hydrolysis with pure enzymes. The units of half-life ($t_{½}$) are min and of k are 10^{-s} min^{-1}. St indicates that the substrate was stable in the presence of this enzyme, and IN, that there were insufficient data points for the kinetic parameter to be determined.

Substrate	CPA		LAP-C		LAP-M	
	$t_{½}$	k	$t_{½}$	k	$t_{½}$	k
FGG	St	St	70	9.91	4	175
WGG	St	St	79	8.80	3	212
GGF	287	2.41	St	St	IN	IN
GF	NT	NT	NT	NT	122	5.67
FG	St	St	567	1.22	88	7.91

The stability of WGG, FGG and FG with CPA is not particularly surprising, in view of the fact that CPA has a number of substrate preferences, one of which is a branched or aromatic amino acid, with L-configuration at the C-terminal end (Kim and Lipscomb, 1990). The order of stability of the substrates with LAP-C is in keeping with the observation that there was variable activity against different substrates. In general, substrates containing an N-terminal leucyl group were preferred, and those with hydrophobic residue at this position were hydrolysed rapidly (Delange and Smith, 1971).

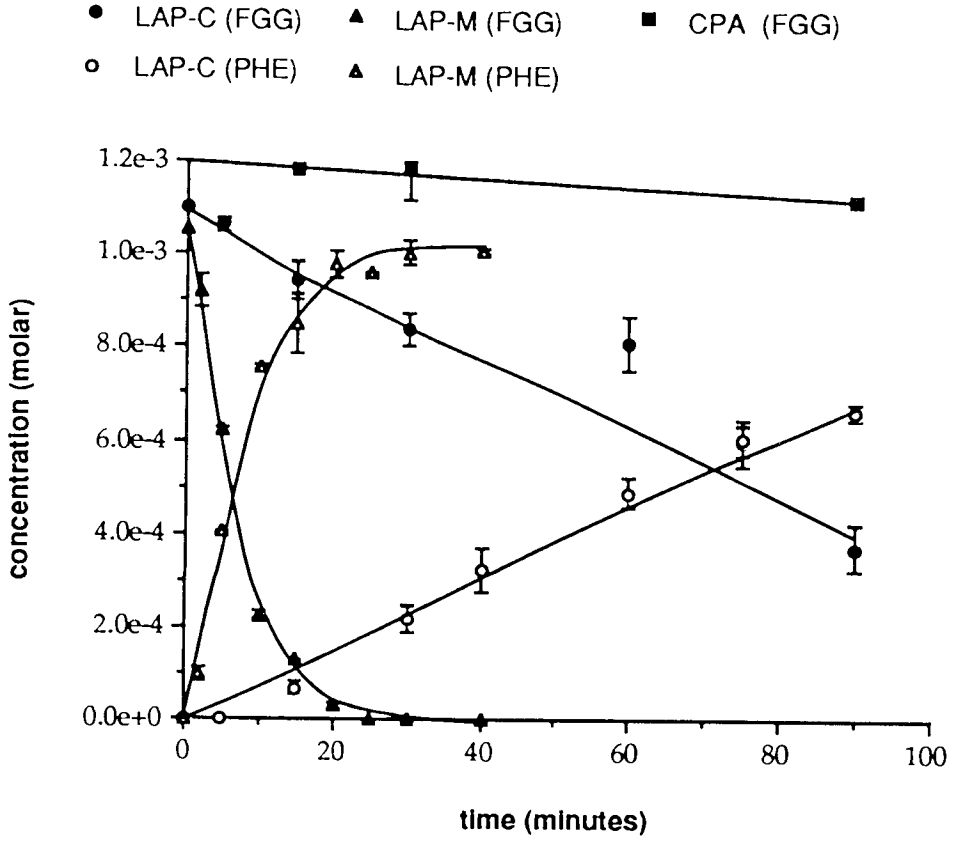
It is difficult to speculate further on the interactions between these substrates and leucine aminopeptidases, because although they are widely distributed cytosolic exopeptidases, which hydrolyse a range of peptides, at the present time the structure and mechanism of action of this class of enzymes are not well characterised. The

crystallographic structure of bovine lens and hog kidney leucine aminopeptidases has recently been reported (Burley et al., 1990; (Taylor et al., 1984) and this may be of use in predicting the susceptibility of substrates to attack by aminopeptidases in the future.

The stabilities of WGG < FGG < FG exhibited by LAP-C and LAP-M are dissimilar to those observed in the sheep nasal homogenate where FGG < FG < WGG section 7.4.2. These differences may reflect that the substrates are being degraded by more than one enzyme in the homogenate, which makes the trend dissimilar. Alternatively, sheep nasal aminopeptidases may have different enzyme preferences to those isolated from porcine kidney.

The hydrolysis profiles for FGG in the presence of the three enzymes are illustrated in figure 7.21.

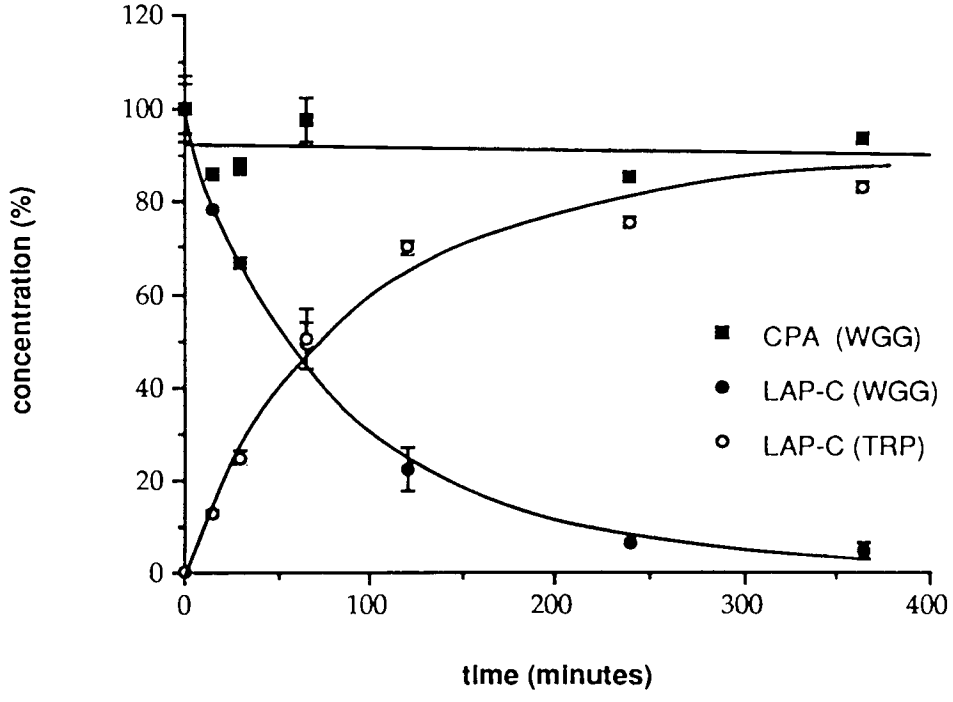
Figure 7.21 The hydrolysis profile of phenylalanylglycylglycine (FGG) in the presence of carboxypeptidase A (CPA), cytosolic leucine aminopeptidase (LAP-C) and microsomal leucine aminopeptidase (LAP-M) in 0.1M phosphate buffer at 37°C. Points are the mean ± sem of two determinations.



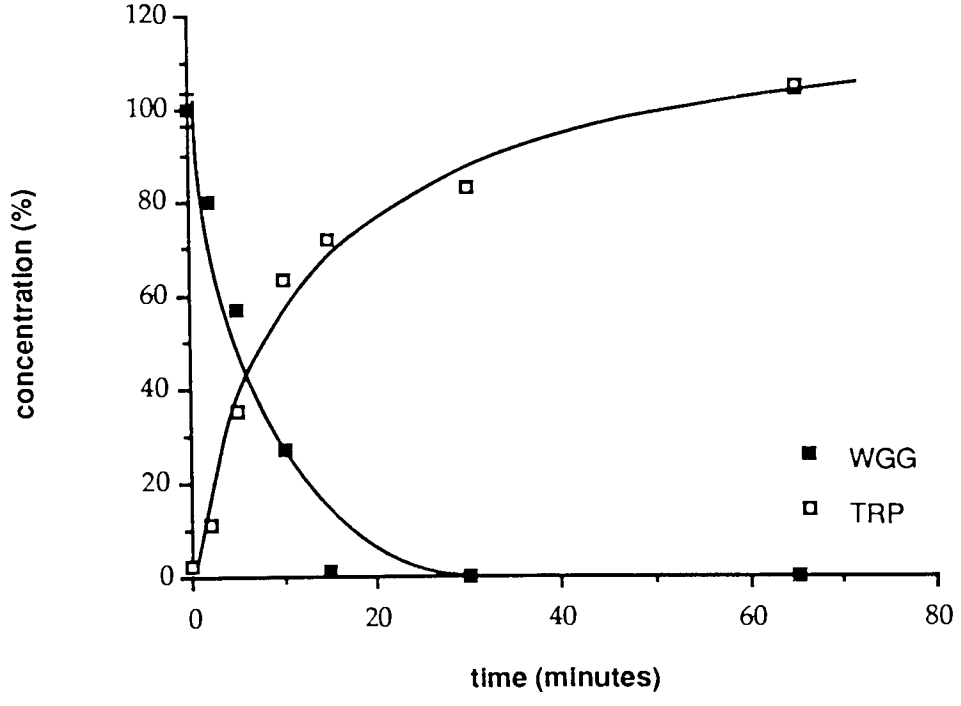
The substrate FGG was stable in the presence of CPA, but was degraded by both LAP-C and LAP-M. For both enzymes attack occurred exclusively at the N-terminal end of the molecule to generate the product PHE. The hydrolysis profiles for WGG are illustrated in figure 7.22. The compound was degraded by the aminopeptidase enzymes by exclusive degradation at the N-terminal end to produce TRP. The product WG was not observed. WGG was stable in the presence of CPA.

Figure 7.22 The hydrolysis profile of tryptophylglycylglycine in the presence of a) carboxypeptidase A (CPA) and cytosolic leucine aminopeptidase (LAP-C) and b) microsomal leucine aminopeptidase (LAP-M) in 0.1M phosphate buffer at 37°C. Points are the mean ± sem of two determinations.

a)



b)



The tripeptide GGF was the only substrate hydrolysed on incubation with CPA. Interestingly, it was additionally the only substrate to be stable in the presence of LAP-C, but was rapidly hydrolysed by LAP-M to G and GF, which was then slowly degraded to PHE and G. The hydrolysis profiles for GGF are illustrated in figure 7.23 and 7.24

Figure 7.23 The hydrolysis profile of glycylglycylphenylalanine in the presence of carboxypeptidase A (CPA) and cytosolic leucine aminopeptidase (LAP-C) in 0.1M phosphate buffer at 37°C. Points are the mean ± sem of two determinations.

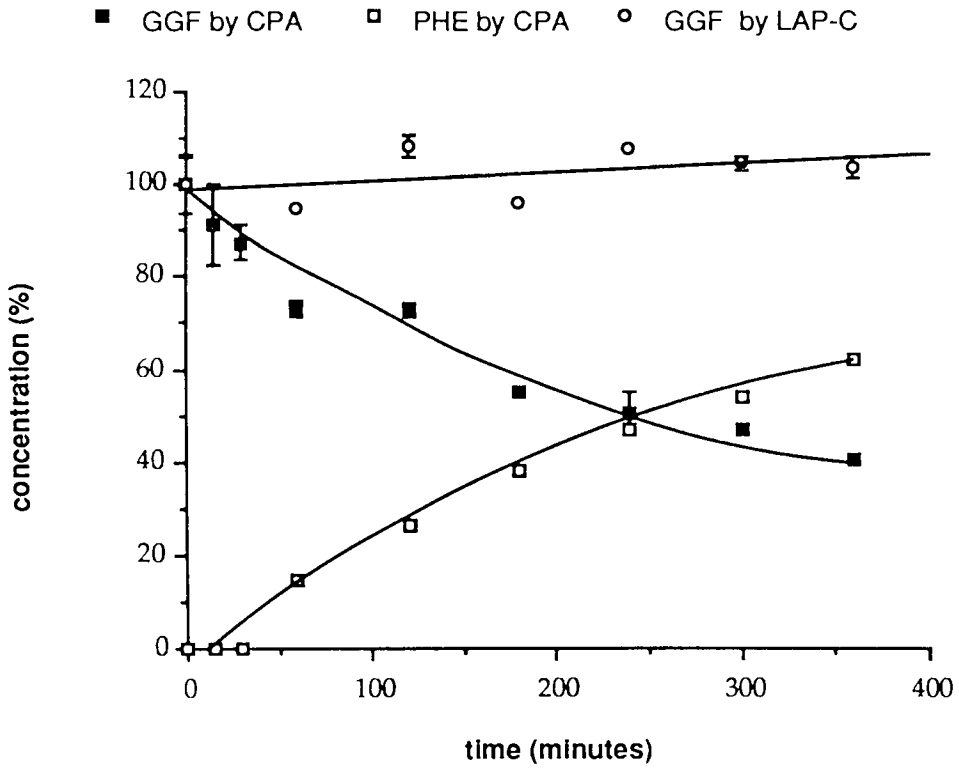
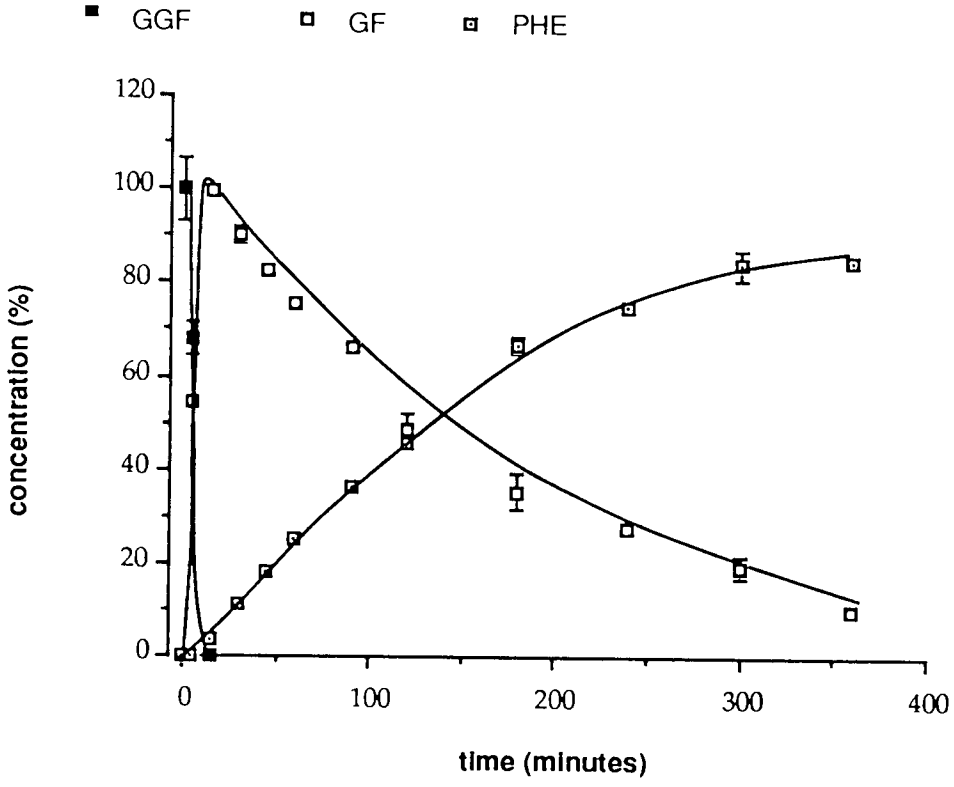
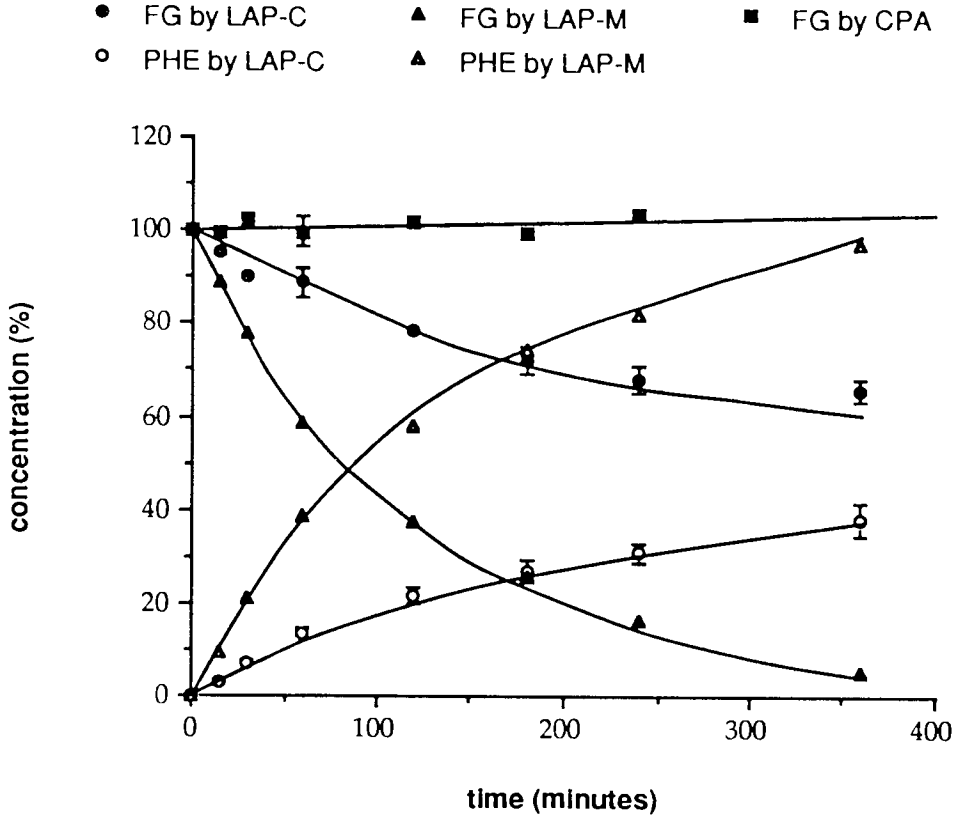


Figure 7.24 The hydrolysis profile of glycylglycylphenylalanine in the presence of microsomal leucine aminopeptidase (LAP-M) in 0.1M phosphate buffer at 37°C. Points are the mean ± sem of two determinations.



The dipeptide FG was stable in the presence of CPA but was degraded by both the aminopeptidases. The hydrolysis profiles for FG are illustrated in figure 7.25. The semilogarithmic plots of time versus concentration for degradation by LAP-C and LAP-M were linear (r = 0.959 and 0.998 respectively), in contrast to the results obtained for the hydrolysis of FG in the homogenate preparation (section 7.4.2), where a concave curve was observed. Similarly, linear plots were also obtained for half-time analysis of the degradation profile of FG by LAP-M, whereas non-linear plots were obtained with the homogenate preparation..

Figure 7.25 The hydrolysis profile of phenylalanylglycine in the presence of carboxypeptidase A (CPA), cytosolic leucine aminopeptidase (LAP-C) and microsomal leucine aminopeptidase (LAP-M) in 0.1M phosphate buffer at 37°C. Points are the mean ± sem of two determinations.

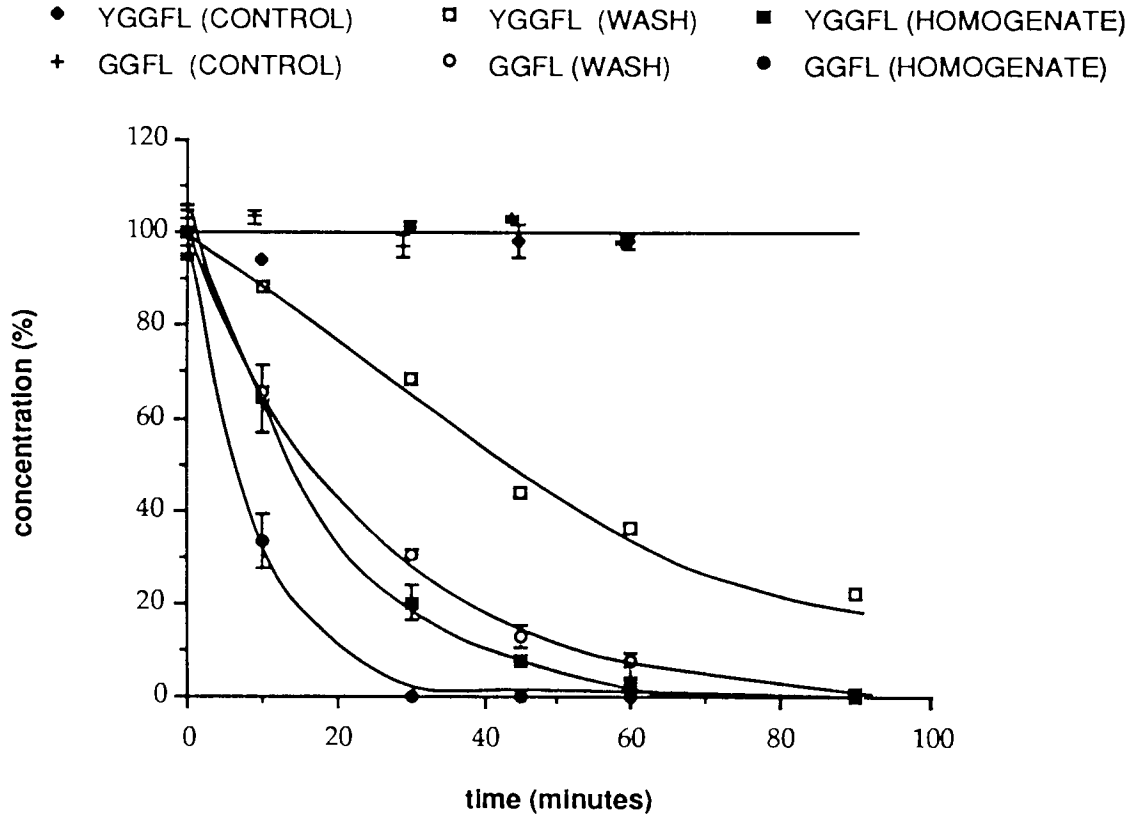


7.4.6 Nasal Wash

Enkephalins are of great interest because of their opiate-like properties and their probable roles as neurotransmitters or neuromodulators. Their physiological effects are very transitory due to their rapid degradation by serum and brain enzymes (Barclay and Phillipps, 1980). In these experiments the hydrolysis of the pentapeptide leucine enkephalin (YGGFL) and the tetrapeptide des-tyrosine leucine enkephalin (GGFL) were monitored in a nasal wash and nasal mucosal homogenate, diluted to the same protein concentration (0.63 mg mL^{-1}). The aim was to investigate and compare luminal and cellular hydrolysis considering both rates and mechanisms of action.

The substrates YGGFL and GGFL were chemically stable in the absence of enzymes but were rapidly hydrolysed in both enzymic preparations exhibiting exponential decline as shown in figure 7.26.

Figure 7.26 The degradation of leucine enkephalin (YGGFL) and des-tyrosine leucine enkephalin (GGFL) using wash and homogenate preparations in 0.1M phosphate buffer at 37°C. Points are the mean ± sem of two determinations.



The half-lives and first-order rate constants were determined from semilogarithmic plots of concentration versus time as described in section 7.2 and are presented in table 7.9.

Table 7.8 First-order kinetic parameters for leucine enkephalin (YGGFL) and des-tyrosine leucine enkephalin (GGFL) in wash and homogenate preparations.

Substrate	Enzymic preparation	k (min^{-1})	$t_{1/2}$ (min)
YGGFL	wash	0.0172	40
YGGFL	homogenate	0.0581	12
GGFL	wash	0.0518	13
GGFL	homogenate	0.106	7

In the wash preparation YGGFL had a half-life of 40 min whilst GGFL was degraded significantly faster with a half-life of 13 min. Hydrolysis in the homogenate preparation was more rapid than in the wash, although the same stability trend for the two substrates was observed, with YGGFL being more stable than GGFL. The half-lives were 12 min and 7 min for YGGFL and GGFL respectively.

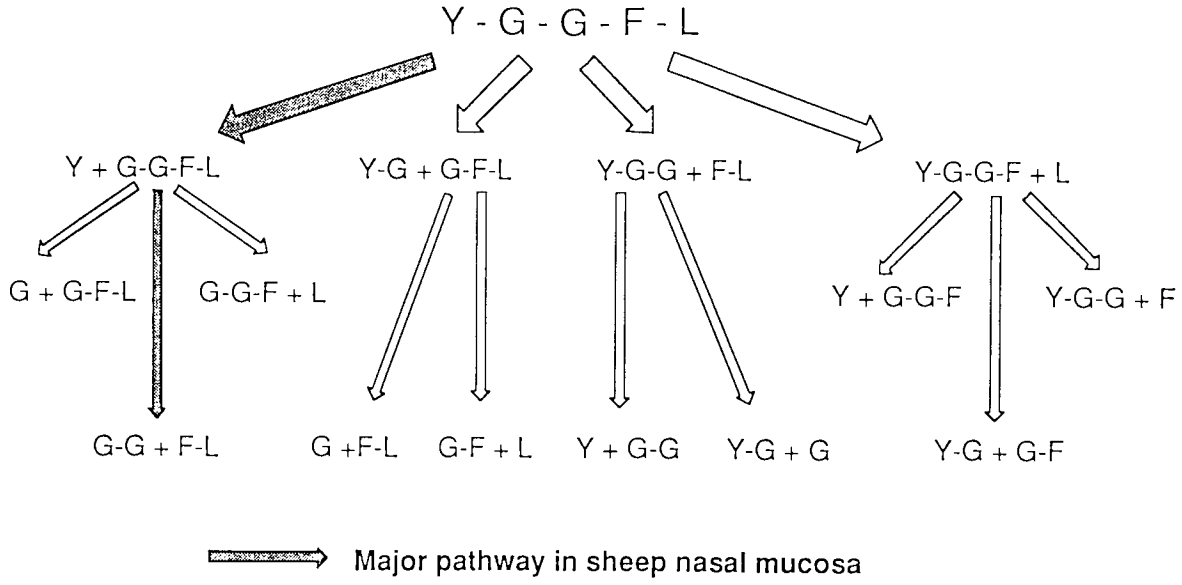
The relative stabilities of YGGFL and GGFL have been investigated in humans, using a wash technique (Hussain et al., 1990). This demonstrated that both substrates were degraded at similar rates. By contrast, experiments using rats and an *in situ* perfusion method found that the initial rate of hydrolysis of YGGFL was twice as fast as YGGFL. This is exactly the opposite to the order observed in sheep.

The activity observed in the wash preparation demonstrates the presence of either loosely bound enzymes, or enzymes which are free in the lumen. Schorn and Hochstrasser (1979) have similarly reported that a number of enzymes, including leucine aminopeptidase, are present in human nasal secretions. The greater activity towards the two peptides in the homogenate compared with the wash may imply that the degradation of these substrates occurs more efficiently with

membrane-bound or intracellular enzymes than by those which are loosely bound or luminal. Alternatively, this trend may simply reflect a higher concentration of proteases in the homogenate preparations, as the protein concentrations determined will not be exclusively due to enzymes. The biological role of these proteases is unclear, although Hussain et al. (1990) has suggested that their purpose may be to defend the upper airways from attack, by degrading inhaled foreign, proteinaceous material.

As can be seen in the schematic diagram in figure 7.27, YGGFL, theoretically could undergo initial hydrolysis at any one of five peptide bonds within its structure simultaneously.

Figure 7.27 Possible hydrolytic pathways for the degradation of leucine enkephalin



In both the wash and homogenate preparations, degradation of YGGFL occurred predominantly by removal of the tyrosine residue at the N-terminal end to yield GGFL. This suggests that aminopeptidases may play a major role in degrading YGGFL. This action is consistent with the results discussed in section 7.4.2 where specificity for the bond between an aromatic bond and glycine was observed.

The tetrapeptide GGFL produced from YGGFL was then further hydrolysed to phenylleucine (FL). This compound was observed on the chromatograms but was not quantified. The products tyrosylgycylglycine (YGG), glycylphenylalanylleucine (GFL) and GGF, which could also be determined using the HPLC protocol described, were not observed. The HPLC traces of the degradation of YGGFL in the wash and homogenate enzymic preparations, which demonstrate this degradation pathway, are shown in figures 7.28 and 7.29.

Figure 7.28 HPLC chromatograms of leucine enkephalin degradation (YGGFL) by the nasal wash at times 0, 30, 60 and 90 min. Experiments were performed in 0.1M phosphate buffer (pH 7.4) at 37°C.

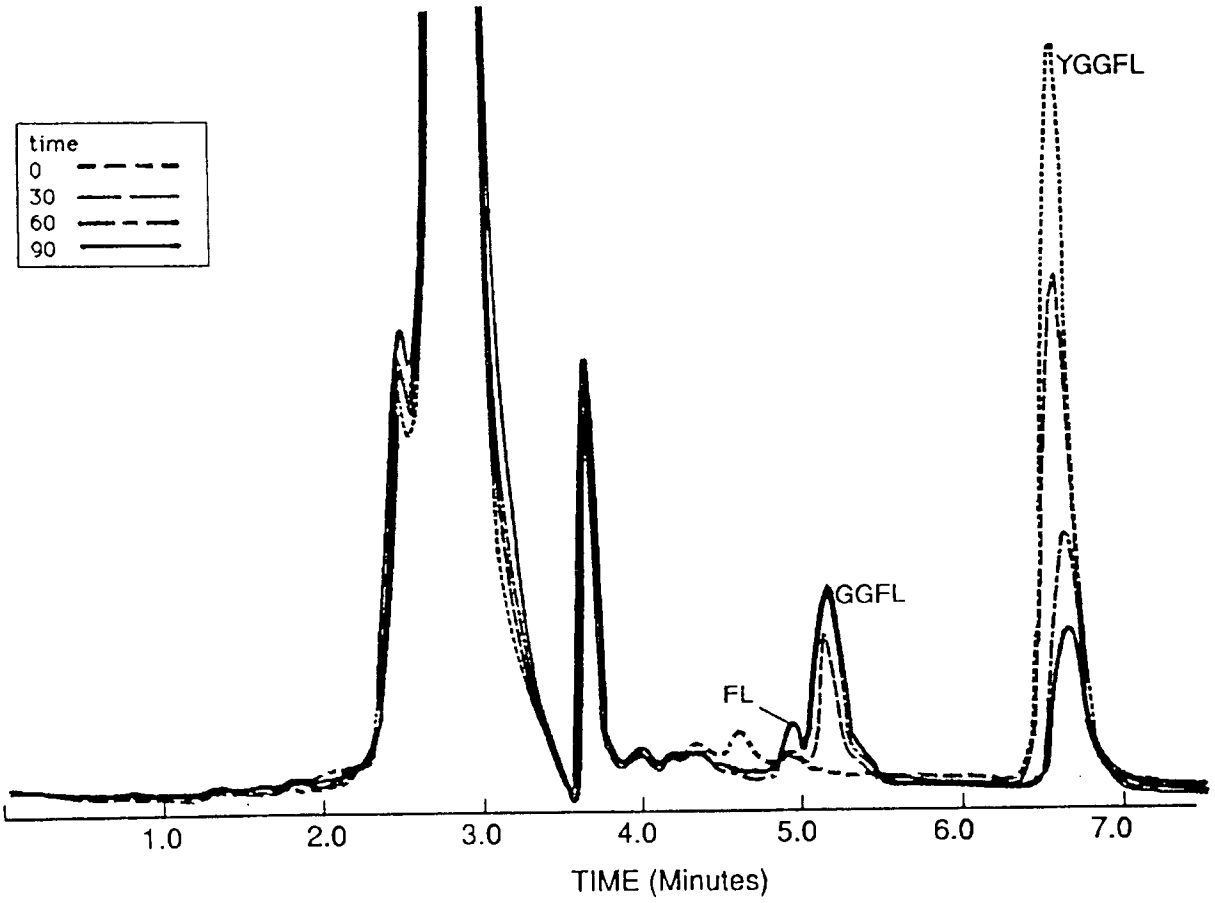
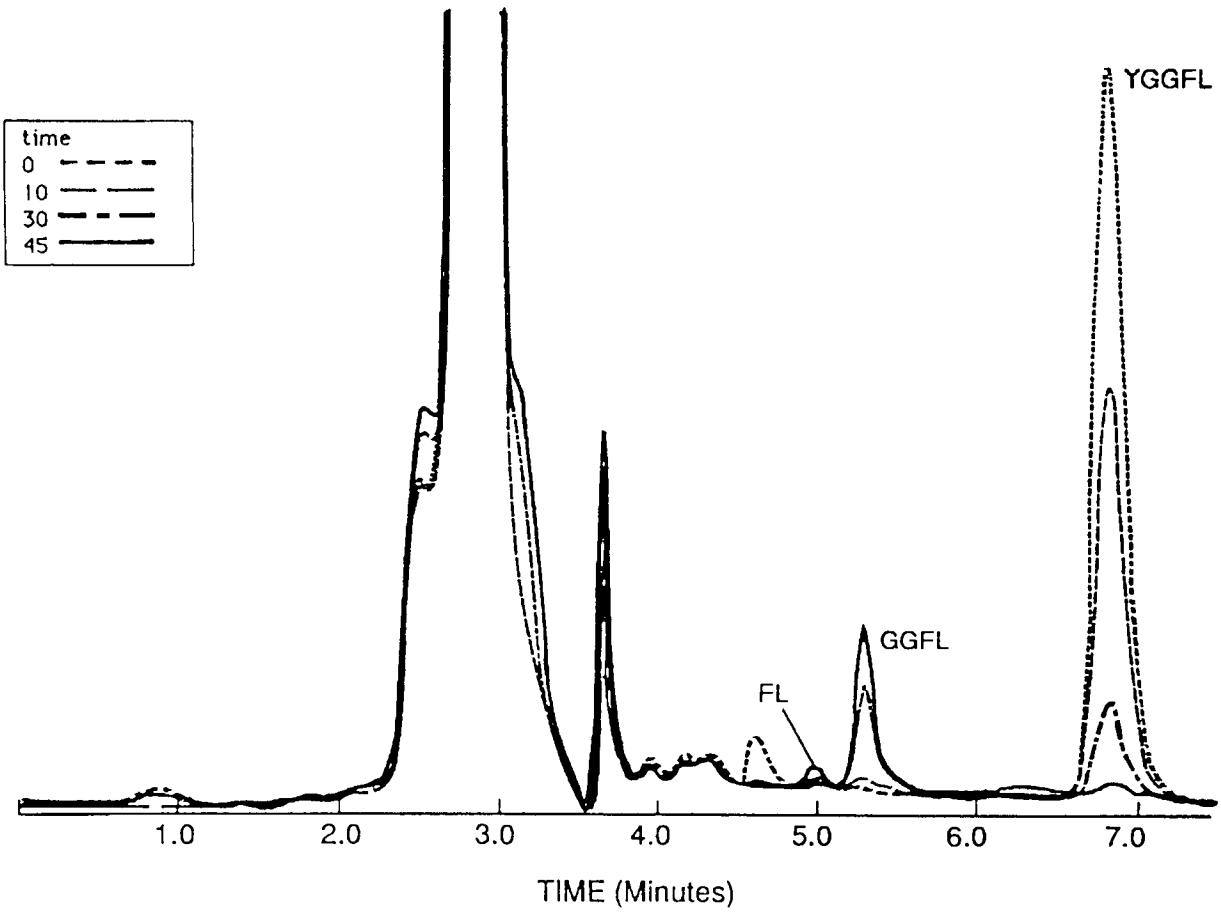
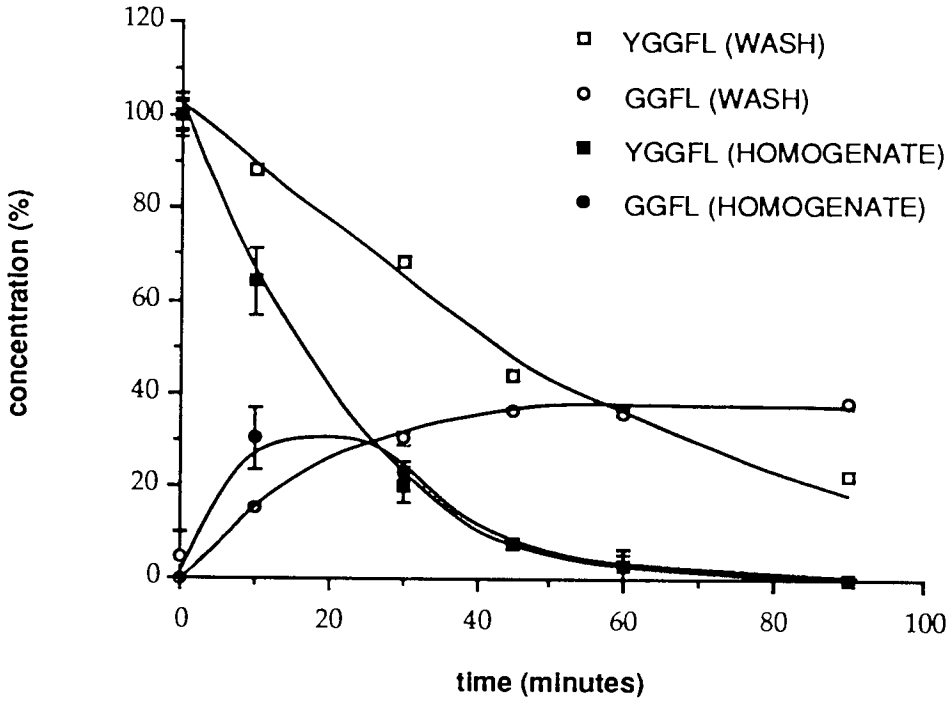


Figure 7.29 HPLC chromatograms of leucine enkephalin degradation by the homogenate preparation at times 0, 10, 30 and 45 min. Experiments were performed in 0.1M phosphate buffer (pH 7.4) at 37°C.



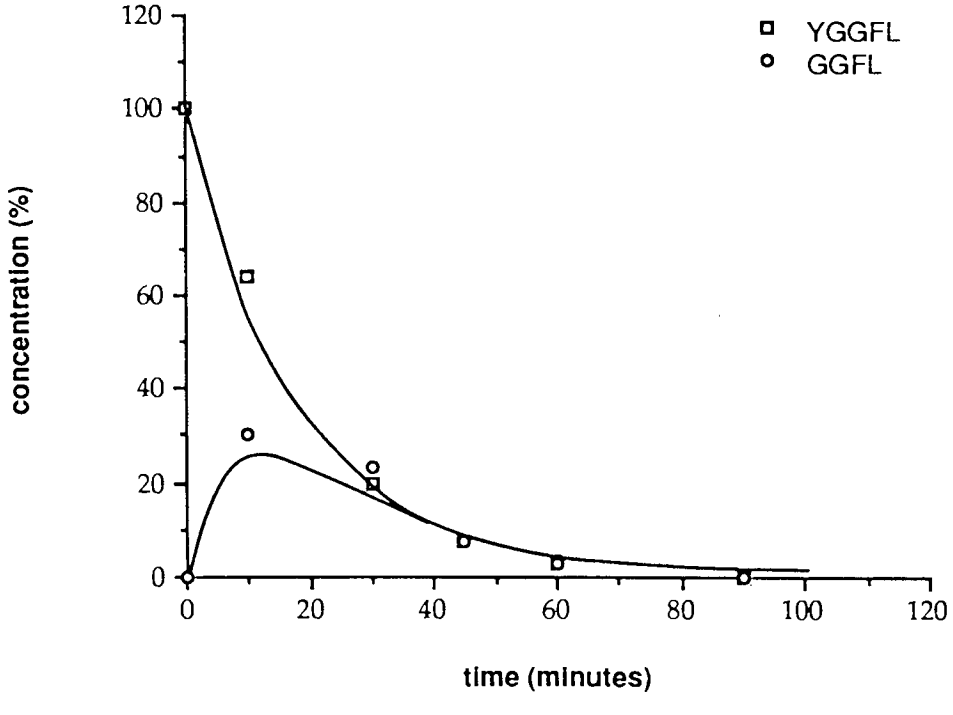
A graph of the hydrolysis profiles of YGGFL and formation of GGFL in both experiments is shown in figure 7.30.

Figure 7.30 Hydrolysis profiles of leucine enkephalin (YGGFL) and formation of des-tyrosine leucine enkephalin (GGFL) in nasal wash and homogenate preparation. Experiments were performed in 0.1 M phosphate buffer (pH 7.4) at 37°C.



To confirm that N-terminal attack was the major route of degradation, theoretical plots were constructed using the first-order rate constants determined experimentally (table 7.9), and the methodology described in section 7.2. These theoretical plots, shown as lines in figure 7.31, confirmed that attack at the N-terminal end of the molecule to generate GGFL was the principal pathway in both the wash and homogenate.

Figure 7.31 Theoretical and experimental profiles of leucine enkephalin (YGGFL) degradation and formation of des-tyrosine leucine enkephalin (GGFL) in nasal homogenate preparation. The symbols represent the mean ± sem of two determinations. The lines represent theoretical values generated by NONREG analysis using the experimentally determined first-order rate constants for YGGFL and GGFL. Experiments were performed in 0.1 M phosphate buffer (pH 7.4) at 37°C.



The metabolism of enkephalin peptides and inhibition of their degradation has attracted considerable attention because of their roles in neurobiology. As a result, the mechanism and rate of YGGFL hydrolysis has been studied in serum and brain tissue. The principal products of YGGFL hydrolysis by brain tissue has indicated that cleavage occurs at the tyrosine-glycine (Y-G) and glycine-phenylalanine (G-F) bond (Fulcher et al., 1982). The former action has been attributed to a membrane-bound aminopeptidase (Barclay and Phillipps, 1980) whilst the latter is catalysed by endopeptidase 3.4.24.11. This is a well-characterised enzyme, which hydrolyses a range of peptides and is widely distributed throughout mammalian tissue (Fulcher et al., 1982; Matsas et al., 1983). It is possible that the same enzymes may be catalysing the reactions in the nasal cavity of the sheep. This would suggest that the hydrolysis of YGGFL to GGFL is a consequence of aminopeptidase action, whilst the production of FL from GGFL may be due to endopeptidase activity. .

The mechanism of YGGFL hydrolysis in the nose of rats, rabbits and humans has also been investigated. In rats, the degradation occurred exclusively at the N-terminal end (Hussain et al., 1985; Faraj et al., 1990a and b) to produce GGFL. Hussain et al. (1985) did not report the presence or concentrations of other metabolites. They confirmed that GGFL was the major product by first-order sequential mathematical modelling of the system with the same equations used in the NONREG analysis (section 7.2). Faraj et al. (1990a and b) similarly monitored only YGGFL and GGFL and confirmed the pathway by theoretical modelling from experimentally determined first-order rate constants.

In rabbits, hydrolysis was observed at three sites, which resulted in the formation of tyrosine (Y), tyrosylglycine (YG) and tyrosylglycylglycine (YGG), with GGFL being the primary product. Further degradation of these metabolites then occurred. In nasal washes taken from humans, in great contrast to the results seen in other animals, GGFL formation was not the major product, but the presence and concentrations of the other metabolites were not given in the communication (Hussain et al., 1990).

The differences in rates and mechanism of degradation of YGGFL suggests that animal species possess different enzymes at a given site or else similar enzymes possessing different relative activities. It emphasises the care needed in choosing an animal model for investigating peptide delivery.

7.5 SUMMARY

While a great deal of information is available about the proteolytic activity of the oral route, the enzymic activity of the nasal route of administration is less well characterised. The aim of this study was to provide more information about the enzymic barrier to peptide drug delivery in the nose. The nasal mucosa of sheep were used as a source of enzymes, and nasal homogenates, cellular fractions and nasal wash preparations were employed to probe the nature and localisation of the activities. Three commercially available proteases were also investigated for comparison.

The results demonstrated that sheep nasal mucosa possesses significant peptide hydrolase activity with a variety of exopeptidases and an endopeptidase capable of hydrolysing di-, tri-, tetra- and pentapeptides as shown by experiments performed with homogenate and wash preparations.

There was a large variation in the stabilities of the substrates in homogenate experiments varying from complete disappearance within ten min in the case of GF, to WG which was still quantifiable after 360 min. The action was mediated by both aminopeptidases and carboxypeptidases. There was some specificity for aromatic amino acids as shown by bonds between Trp-Gly, Phe-Gly, Gly-Phe and Tyr-Gly being broken, the reason for this is however unclear.

The aminopeptidase activity per mg of protein was greater in the microsomal preparation than that in the cytosolic preparation towards GGF. Greater microsomal activity per mg of protein was also observed with the pure aminopeptidases LAP-C and LAP-M against FGG, WGG, GGF and FG.

The activity of the nasal mucosal homogenate against YGGFL and GGFL was significantly greater than that observed with the wash. This may reflect greater intracellular or membrane-bound activity towards these substrates compared to luminal or loosely-bound enzymes. Alternatively, it may simply reflect a higher concentration of proteases in the homogenate mixture. YGGFL was degraded predominantly by aminopeptidase action to GGFL which was then hydrolysed to GG and FL, possibly by an endopeptidase, in both enzymic preparations. The mode of degradation was similar to that seen in rats by other workers, but in contrast to the reported results in humans. The relative stabilities of YGGFL and GGFL were also dissimilar to those observed in rats and humans.

CHAPTER 8
GENERAL SUMMARY

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GENERAL SUMMARY

In the last few years it has become apparent that many of the drugs of the future will be proteins or peptides, discovered or developed through recombinant DNA technology (Sadee, 1986). A recent review has stated that, worldwide, over ninety human therapeutic proteins are being developed commercially as potential drug candidates (O'Hagan and Illum, 1990). It has also become evident that a major limiting factor in the exploitation of these drugs is the lack of appropriate ways to deliver them to the patient.

Intranasal administration has attracted attention as being a viable alternative to parenteral delivery, and has been successful for the delivery of some bioactive peptide and protein molecules. In order to optimize drug delivery to the nasal cavity it is necessary to define the nature and relative contribution of the barriers which restrict absorption from the nose. For this reason it was decided to investigate the role of two of the biological barriers, the mucus layer and enzymic degradation, in peptide drug delivery.

Pig gastric mucus (PGM) was selected as a model secretion because of its ready availability, the knowledge of its chemical composition and rheological properties, and the difficulty in obtaining sufficient quantities of normal human nasal secretions. A method of preparing and storing the mucus was validated with respect to the biochemical composition of the secretion (water, protein and hexose) and to its rheological properties (Chapter 3).

The nature of the barrier that the mucus layer presents to drug molecules was investigated using side-on three-compartment diffusion cells (chapter 4). The mucus was contained between two nuclepore polycarbonate membranes which were inserted between a donor (drug containing) compartment and a receiver (drug free) compartment. The effect of increasing molecular weight, charge, hydrophilicity and chemical group (characteristic features of peptide molecules), were investigated with the compounds: tryptophan (TRP), tryptophylglycine

(WG) and tryptophylglycylglycine (WGG); benzoic acid (BA), β-blockers (propranolol (PR), oxprenolol (OX) and atenolol (AT)) and N-acetylphenylalanine (NP) respectively.

All of the compounds evaluated were able to penetrate the mucus layer, although the rates of diffusion were significantly lower than the results obtained for an unstirred aqueous layer of the same thickness. The experimental results suggested that the reduction in diffusion rate was a consequence of mechanical obstruction, as indicated by molecular weight, and chemical interaction between the drug molecules and the mucus gel. Extrapolating the results for the effect of molecular weight would imply that the transport of larger peptidic molecules through mucus may be more difficult, due to greater mechanical obstruction. McMartin et al. (1987) reported a molecular weight cut off value of 1000 for the successful nasal delivery of peptides. Whether this observation relates to exclusion by the mucus layer needs further experimental validation.

The effect of absorption enhancing agents (which possessed the ability to alter mucus structure) upon the rate of diffusion of TRP was investigated using three-compartment diffusion cells (chapter 5). The enhancers evaluated were taurocholic acid (TA), taurodeoxycholic acid (TDA), cholic acid (CA), ethylenediaminotetraacetic acid (EDTA) and N-acetylcysteine (NAC). The enhancers TDA, EDTA and NAC did not exert any effect upon the diffusion rate at the concentrations employed. Addition of TA and CA to the donor compartment slightly reduced the rate of diffusion. Direct mixing of TA with mucus immediately prior to diffusion experiments produced longer lag times and slower diffusion rates.

Drug-mucus binding effects were investigated using the centrifugation method of Saitoh et al. (1986), and the compounds utilised in the diffusion experiments (chapter 6). Klotz, Scatchard and Freundlich plots of the binding data indicated the presence of binding sites with differing affinities for the molecules. All of the compounds, with the exception of the negatively charged benzoic acid, bound to

mucus. The adsorbent capacity and intensity of binding to mucus was determined from the Freundlich adsorption isotherm. The greatest adsorbent capacity and intensity of binding were obtained for the positively charged β-blockers, indicating the importance of electrostatic interactions. This was also implicated from the lower adsorbent capacity and binding intensity of NP, in comparison with TRP. An investigation of the relationship between mucus-binding and diffusion through mucus produced conflicting results.

The intracellular and luminal proteolytic activity of the nose was investigated using tissue homogenate and nasal wash preparations taken from sheep nasal mucosa, and a series of chemically similar peptides ranging from di- to pentapeptides (chapter 7). The hydrolysis profiles of the peptide substrates were also determined in the presence of three commercially available proteolytic enzymes: carboxypeptidase A, cytosolic leucine aminopeptidase and microsomal leucine aminopeptidase.

All of the substrates were chemically stable in the absence of enzymes. In contrast, in the homogenate preparation they were all hydrolysed exhibiting a considerable range of stabilities. Hydrolysis was performed by carboxypeptidase enzymes, which have not previously been demonstrated in sheep nasal mucosa, and aminopeptidases. Specificity was observed for the bond between glycine and aromatic amino acids. The use of cellular fractions, prepared from the mucosal homogenate demonstrated that microsomal activity was greater than cytosolic activity.

Peptide hydrolase activity was also observed in the nasal wash preparation, against leucine enkephalin (YGGFL) and des-tyrosine leucine enkephalin (GGFL), indicating the presence of luminal or loosely-bound proteolytic enzymes. The same specificity for aromatic amino acids was observed as for the homogenate. The reactions were mediated by an aminopeptidase, which may be leucine aminopeptidase, and an endopeptidase. The activity of the nasal mucosal homogenate against YGGFL and GGFL was significantly greater than that observed

with the nasal wash, although the same degradation pathways were followed.

It has been proven that mucus has the ability to retard diffusion and to bind a number of molecules, including peptide molecules. For the small peptidic molecules studied it would appear that mucus is not the major barrier to absorption, and is not as important as the enzymic barrier, as the molecules can penetrate the mucus gel in appreciable quantities. The negative effect of the various enhancers on the diffusion rate *in vitro* also implied that the mucus layer is not the greatest problem to successful delivery. This barrier may, however, assume a greater role with larger, higher molecular weight species. The situation with larger molecular weight peptidic molecules was not assessed due to financial constraints.

The results of this study demonstrate the wide variation in the types and distribution of enzyme activities in the nose and indicate the considerable barrier that enzymic degradation presents to peptide drug delivery. Indeed, Lee (1988) has suggested that the enzymic barrier is more of an impediment to insulin absorption than the membrane transport barrier. The distribution and diversity of enzymes makes it unlikely, as yet, to be able to predict the degree to which enzymically-labile drugs might be absorbed across the nasal mucosa. Further work is needed to characterise the enzymic barrier in the nose with respect to assigning the major site of degradation and the enzymes responsible for the deactivation. For example, in the GI tract it has been determined that peptides are initially hydrolysed in the lumen to smaller subunits. The smaller subunits are then degraded by the brush border enzymes, which are anchored in the apical membrane, but have their active sites in essentially an extracellular environment. Such information could lead to the development of more appropriate formulations, to protect the peptide molecule.

An additional factor, which has not been addressed to date, is the role of bacterial proteases in degrading peptide molecules introduced into the nose. It is a recognised fact that in the normal human nose,

nearly 50% of the population contain staphylococcus, in smaller or greater amounts (Tremble, 1949). While it has been acknowledged that bacterial flora in the colon may influence drug delivery, this fact has not been addressed in nasal administration. This factor may be important if microrganisms in the nose release proteolytic agents in a similar way to those in the gut.

The nasal route of administration offers many challenges to the pharmaceutical scientist to develop reliable, safe and commercially acceptable dosage forms for peptide and protein molecules. Future success may include the usage of safe, non-toxic absorption enhancers, based on biological agents such as phospholipids and lung surfactant. Improved formulations, such as mucoadhesion may additionally lead to reduced inter- and intra-subject variability and improved bioavailabilities. An extremely novel suggestion is that genetic engineering could be used to produce humans with large noses to facilitate intranasal absorption of drugs!

However, although the need for suitable delivery systems for peptides is evident and the advantages of nasal delivery stated it appears that it may be some time before the following citation by Davis (1986) becomes a reality:

> "The way in which the drug would be ingested ... was important. We've researched this exhaustively and recommend delivery by nasal spray. This is the modern coming system.... Peptide 7 will be in an inert saline solution mixed with a detergent (that) assures the best absorption rate.... The best non-toxic (detergent) creating no irritation of nasal membranes had been found."

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APPENDIX 1

LIST OF SUPPLIERS

Chemical	Supplier
Acetonitrile	FSA Laboratory supplies
N-Acetylcysteine	Sigma
N-Acetyl-L-phenylalanine	Sigma
Atenolol	Sigma
Benzoic acid	Aldrich
Bovine serum albumin	Sigma
Brilliant blue G-250	Sigma
Carboxypeptidase A	Sigma
Cholic acid (sodium salt)	Sigma
Citric acid	Fisons
Des-tyrosine leucine enkephalin acetate salt	Sigma
Diethylamine	BDH
Dimethylsulphoxide	BDH
EDTA	BDH
Ethanol	Fisons
Ethyl paraben	Sigma
D-Glucose	BDH
Glycine	Fisons
Glycylglycine	Sigma
Glycylglycylglycine	Sigma
Glycylglycylphenylalanine	Sigma
Glycylphenylalanine	Sigma
Glycylphenylalanylleucine	Sigma
1-Hexane sulphonic acid (sodium salt)	BDH
p-Hydroxybenzoic acid	Sigma
K_2PO_4	Fisons
Leucine aminopeptidase (cytosolic)	Sigma
Leucine aminopeptidase (microsomal)	Sigma
Leucine enkephalin acetate salt	Sigma
Methyl paraben	Sigma

Na_2HPO_4	Fisons
$Na_2HPO_4 . 2H_2O$	BDH
$NaH_2PO_4 . 2H_2O$	Fisons
1-Octanesulphonic acid	Sigma
Orcinol	Sigma
Oxprenolol	Sigma
Phenylalanylglycine	Sigma
Phenylalanylglycylglycine	Signa
Phenylalanylglycylglycylphenylalanine	Sigma
Phenylalanylleucine	Sigma
L-Phenylalanine	Sigma
L-Phenylalanine methyl ester	Sigma
L-Phenylalaninol	Sigma
Phenylmethylsulphonylfluoride	Sigma
Phenylalanylphenylalanine	Sigma
Phenylalanylphenylalanylphenylalanine	Sigma
o-Phosphoric acid	Fisons
Potassium chloride	Fisons
DL-Propranolol	Sigma
Salicylic acid	BDH
Sodium Azide	Sigma
Sodium Chloride	Aldrich
Sodium Hydroxide	BDH
Sodium Lauryl sulphate	Fisons
Tartaric acid	BDH
Taurocholic acid (sodium salt)	Aldrich
Taurodeoxycholic acid (sodium salt)	Sigma
Trifluoroacetic acid	Aldrich
Tryptophan 3H	Du Pont
Tryptophylglycine	Sigma
Tryptophylglycylglycine	Sigma
Tryptophylphenylalanine	Sigma
L-Tryptophan	BDH Biochemicals

APPENDIX 2

COMPOSITION OF BUFFERS

Buffer compositions were taken from Perrin and Dempsey (1974).

Phosphate buffer

Contains x mL 0.2M Na_2HPO_4 (28.39 g L^{-1}) and (50-x) mL 0.2M $NaH_2PO_4.2H_2O$ (31.21 g L^{-1}) diluted to 100 mL.

pH	x	pH	x
5.8	4.0	7.0	30.5
6.0	6.15	7.2	36.0
6.2	9.25	7.4	40.5
6.4	13.25	7.6	43.5
6.6	18.75	7.8	45.75
6.8	24.5	8.0	47.35

McIlvaine's buffer

Contains x mL 0.1M citric acid (21.01 g $C_6H_8O_7.H_2O$ L^{-1}) and (100-x) mL 0.2M disodium hydrogen phosphate (35.61 g $Na_2HPO_4.2H_2O$ L^{-1}). KCl was added to produce an ionic strength of 0.5M.

pH	x	KCl added (g/100 mL)	pH	x	KCl added (g/100 mL)
3.4	72.4	2.83	5.6	42.6	1.49
3.6	68.7	2.72	5.8	40.2	1.42
3.8	65.2	2.61	6.0	37.5	1.34
4.0	61.9	2.46	6.2	34.6	1.19
4.2	59.0	2.31	6.4	31.1	1.12
4.4	56.3	2.20	6.6	27.1	1.04
4.6	53.8	2.09	6.8	22.8	0.969
4.8	51.4	1.94	7.0	17.8	0.746
5.0	49.0	1.86	7.2	13.0	0.522
5.2	47.0	1.71	7.4	9.4	0.298
5.4	44.8	1.64	7.6	6.5	0.075

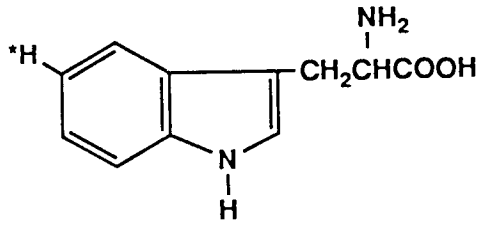
APPENDIX 3

TECHNICAL DATA FOR RADIOLABELLED TRYPTOPHAN

Name and catalogue number: NET-782 TRYPTOPHAN, L-[5-^{3}H]-

Supplier : New England Nuclear (NEN)

Chemical structure



Molecular weight	:	204.2
Specific activity	:	1165.5 GBq/mmol (31.5 Ci/mmol)
Concentration	:	37.0 MBq/mL (1.0 mCi/mL)
		(0.032 µmol/mL)
		(0.0065 mg/mL)
Radiochemical purity	:	99%
		determined by HPLC and paper chromatography
Optical purity	:	99%
		determined on a Crownpak CR chiral HPLC column