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Enhancement of intestinal absorption of peptides

A thesis submitted for the degree of
Doctor of Philosophy

by

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Neuroscience and Biomedical Systems
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I am tired of this sort of thing called science. We have spent millions in that sort of thing for the last few years, and it is time it should be stopped.

Senator Simon Cameron, 1861

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SUMMARY

1. Peptide hormones, once available only through extraction from animal tissue, are now increasingly manufactured synthetically. However, the route of administration is usually by injection due to the low oral bioavailability of the hormones, as a consequence of proteolytic degradation in the small intestine, poor penetration of the intestinal mucosa, and clearance from the portal vein by the hepatocytes of the liver.

2. Since bile acids are efficiently absorbed by an active carrier system in the ileum, the present study investigated the degree to which transport of ileally-administered tetragastrin, a peptide composed of four amino acid residues, could be enhanced by coupling with cholic acid. Conjugation with cholic acid was at the position where glycine and taurine attach naturally in the formation of conjugated bile salts.

3. In the fasted anaesthetised rat, gastric acid secretion was measured at 15 min intervals as a bioassay for the levels of tetragastrin in the blood stream. In all experiments, intravenous administration of tetragastrin or the tetragastrin-cholic acid conjugate (G-CA) was adopted as the first and final procedure, in order to demonstrate the continued viability of the animal preparation.

4. Intravenously administered tetragastrin (minimum effective dose, most commonly 15μg kg^{-1}) as a first procedure was shown to evoke a mean peak increase in gastric acid levels of 0.50 ± 0.06μmol $15min^{-1}$. Over a period of one hour following injection, a mean cumulative increase in gastric acid output of 1.07 ± 0.39μmol hr^{-1} was demonstrated (n=31). G-CA (15μg kg^{-1}) also demonstrated biological activity when administered intravenously: a mean peak increase in gastric acid levels of 0.50 ± 0.18μmol $15min^{-1}$, and a mean cumulative increase of 0.91 ± 0.47μmol hr^{-1} were obtained (n=32). In both cases, a broadly comparable increase in gastric acid secretion was also obtained in response to a second intravenous injection, indicating that the animal preparation was neither fatigued, nor that earlier procedures had resulted in potentiation of the response.

5. Intra-intestinal administration of tetragastrin (2500μg kg^{-1}) was shown not to produce a measurable increase in gastric acid levels. By contrast, the present study demonstrated unequivocally that infusion of G-CA (600μg kg^{-1}) into the ileum resulted in the stimulation of increased levels of gastric HCl secretion (means of small samples were weighted to give a true mean increase of 1.84 ± 1.22μmol $180min^{-1}$).

6. Simultaneous infusion of tetragastrin and glycocholic acid (in a ratio of 3:2, total dose of 600μg kg^{-1}) into the ileum did not elicit any measurable increases in gastric acid secretion, despite the solution being biologically active, as confirmed by a measurable increase in gastric acid secretion following intravenous injection (1.09 ± 0.58μmol hr^{-1}, P=0.033; n=5).

7. Surprisingly, instillation of tetragastrin (2500μg kg^{-1}) into the ileum did evoke a significantly marked increase in gastric acid secretion (3.05 ± 1.89μmol $180min^{-1}$, P=0.006; n=7), though only when tetragastrin was preceded by ileally-administered G-CA (600μg kg^{-1}). This indicated that a possible lasting permeability change in the ileal mucosa was caused by ileal G-CA, thus permitting the permeation of the intestinal wall by tetragastrin. Nevertheless, the results of light microscopic analysis revealed little evidence of a pathological alteration to the intestinal mucosa.

8. The transmucosal movement of G-CA appeared to be specific to the ileum, as there was no evidence of transport across the jejunal mucosa following intra-luminal instillation of G-CA (600μg kg^{-1}). Subsequent administration of G-CA (600μg kg^{-1}) in the ileum of the same rat produced a significant increase in gastric acid secretion.

9. The results of the present study are indicative of utilisation of the bile salt active transporter by G-CA. This is very encouraging in terms of a successful approach to address the facilitation of permeation of otherwise poorly absorbed oligopeptides across the intestinal mucosa and into the systemic circulation.

INTRODUCTION

The self-evident drawback of drug delivery by injection reinforces the preference for the oral route for the administration of therapeutic drugs. However, oral delivery of various drugs, particularly peptides, very often results in a relatively poor bioavailability (the proportion of the intact biologically active compound that eventually reaches the systemic circulation from the site of administration), as a result of degradation by proteolytic enzymes both in the gastrointestinal lumen and during absorption through the intestinal epithelium, poor penetration of the intestinal mucosa and extensive first-pass metabolism by the liver (Doherty and Pang, 1997).

1.1 Overview of protein digestion and absorption

In order to understand the difficulties involved in achieving success in oral delivery of peptide drugs, it is important to provide a general review of protein digestion and absorption. A more detailed account can be found by referring to the chapters by Alpers (1994) and Ganapathy, Brandsch and Leibach (1994).

1.1.1 Pepsin

Protein digestion within the gastrointestinal tract begins in the stomach. Pepsin, the active protease, is thought to be secreted mainly by the chief cells in its inactive form, pepsinogen. The presence of hydrochloric acid converts pepsinogen to pepsin, and also provides an optimal pH of 1.5 to 3.5 for the activity of the enzyme. Pepsin then, by the process of autocatalysis, carries the conversion of pepsinogen to pepsin to completion (chapter by Bell, Emslie-Smith and Paterson, 1976). Proteins most susceptible to pepsin-catalysed hydrolysis are those that contain peptide bonds formed by amino acid residues (of L-configuration) containing aromatic side chains (Mahler and Cordes, 1969a). Pepsin is referred to as an endopeptidase since it hydrolyses peptide bonds in the interior of polypeptide chains (Mahler and Cordes, 1969a). The action of pepsin breaks proteins down into a mixture of peptides of varying chain lengths (referred to as peptones in older texts).

1.1.2 Pancreatic proteases

The next stage of protein hydrolysis occurs in the duodenum and small intestine, and involves the pancreatic proteases, namely trypsin, α-chymotrypsin, elastase and carboxypeptidases A and B (Lee and Amidon, 1995). The actions of the pancreatic endopeptidases, trypsin, α-chymotrypsin and elastase are complementary in that they catalyse the hydrolysis of almost all of the internal peptide linkages likely to be encountered in proteins and peptides. Trypsin cleaves peptide bonds at basic amino acids such as lysine and arginine; α-chymotrypsin cleaves peptide bonds at hydrophobic amino acids such as leucine, methionine, phenylalanine, tryptophan and tyrosine; elastase acts on the peptide bonds of amino acid residues with smaller, unbranched, non-aromatic structures such as alanine, glycine, isoleucine, serine and valine. Carboxypeptidases are exopeptidases which act on substrates with a free terminal carboxyl group and a C-terminal amino acid of L-configuration (Lee, 1988). The summed actions of these enzymes results in the production of approximately 40% free amino acids and 60% peptides with two to six amino acids (Alpers, 1994), although the extent of luminal degradation of proteins and peptides is determined by both the size of the protein molecule and the amino acid composition of these compounds. Adibi and Morse (1977) demonstrated that, within the human jejunal lumen, di- and triglycines were not hydrolysed; however, tetra- to hexaglycines did undergo degradation. Further, Adibi (1971) also found that although di- and triglycines were not hydrolysed in the human small intestine, di- and trileucines were.

1.1.3 Brush border and intracellular peptidases

The most important peptide-hydrolysing agents appear to exist at the brush border membrane and within the cytosol of the enterocytes. Listings of the known brush border and intracellular peptidases, to date, may be found in a review by Alpers (1994), while, a simplified version is reproduced in Tables 1-1a and 1-1b. There may also be further degradative activity as a result of pancreatic proteases such as trypsin and chymotrypsin, adsorbed from the luminal fluid on to the enterocyte brush border (Goldberg, Campbell and Roy, 1968; 1971). The brush border peptidases are capable of hydrolysing peptides of up to ten amino acid residues, but have a preference for tripeptides rather than dipeptides (Matthews and Payne, 1980).

Table 1-1a: Intestinal brush border peptidases

Enzyme	Substrate
Neutral endopeptidase	Hydrophobic internal amino acids
Aminopeptidase N	Oligopeptides C_2-C_6 with neutral AA-X-
Aminopeptidase A	Oligopeptides C_2-C_6 with Asp/Glu-X-
Aminopeptidase P	Oligopeptide with X-Pro-
Aminopeptidase W	Oligopeptide with Glu-Trp-
Dipeptidyl aminopeptidase IV	Oligopeptides with Pro-X- or Ala-X-
Dipeptidyl carboxypeptidase (ACE)	Angiotensinogen or -X-Pro-
Carboxypeptidase M	Peptides with -X-Lys- or -X-Arg-
γ-Glutamyl transpeptidase	Peptides with γ-glutamyl bonds
Carboxypeptidase P	-X-Pro-
Folate conjugase	Polyglutamyl folates
Membrane Gly-Leu peptidase	Dipeptides, especially Gly-Leu
Zinc-stable Asp-Leu dipeptidase	Dipeptides, especially Asp-Leu
Membrane peptidase	Dipeptides and glutathione conjugates

(AA, amino acids; -X-, next amino acid in sequence; ACE, angiotensin converting enzyme; Asp, aspartate; Glu, glutamate; Pro, proline; Trp, tryptophan; Ala, alanine; Lys, lysine; Leu, leucine)

Table 1-1b: Intestinal intracellular peptidases

Enzyme	Substrate
Amino tripeptidase	Tripeptides
Amino dipeptidase	Dipeptides
Prodipeptidase	X-Pro dipeptides
Arg-selective endoproteinase	Presomatostatin -Arg-X-

(-X-, next amino acid in sequence; Pro, proline; Arg, arginine)

1.2 Intestinal transport of amino acids and peptides

The intestinal epithelium has efficient transport mechanisms to absorb not only free amino acids from the gastrointestinal lumen, but also di- and tripeptides. Free amino acids are absorbed into the enterocyte across the brush border membrane via group-specific amino acid transport systems. Di- and tripeptides are transported intact across the brush border membrane via specific peptide transport systems (Newey and Smyth, 1960; Newey and Smyth, 1962). These peptides undergo rapid hydrolysis by cytosolic peptidases to produce free amino acids (see Table 1-1b). The egress of amino acids from the enterocyte is via amino acid transport systems, although a very small proportion of intact peptides may also enter the portal circulation (Ganapathy, Brandsch and Leibach, 1994).

It is worth mentioning at this point that the small intestine of the neonatal mammal differs from the adult small intestine in its increased capacity to take up and transport proteins (Walker, 1979). This process appears to be initiated by the adsorption of large molecules, for example, immunoglobulins on to the microvillous membrane of enterocytes. Once in sufficient concentration, invagination of the membrane occurs and small vesicles within the cytoplasm are formed. After a period of time, when intestinal immunological and non-immunological host defences have developed, the rate of uptake of macromolecules diminishes. This is the process known as "closure", and is the result of morphological and functional maturation of the small intestinal epithelial cells. In rats, closure occurs 20 days after birth. In man the process is more subtle, although it is now well established that the intake of human milk by the new-born facilitates the maturation of the intestine, leading to "closure" (Walker, 1979).

It is clear, therefore, that in order to increase the bioavailability of orally administered peptide drugs, the enzymatic barrier must be by-passed in some way. Reducing proteolytic degradation may be achieved by a number of strategies, for example, by modification of the peptide structure, by use of protease inhibitors, or by using the formulation approach to protect the peptide against proteolytic attack. These ideas will be discussed later in more detail.

1.3 Anatomical barriers to intact peptide absorption

The wall of the gastrointestinal tract acts as a physical barrier to the absorption of intact peptide drugs, and is a major factor to be taken into consideration if a method of oral administration is to be achieved.

The stomach is of little significance in terms of absorption; the small intestine, the major site of food and drug absorption, is far more important, and its anatomical structure will thus be described. For a more detailed account, reviews by Carr and Toner (1984), Kararli (1989) and Madara and Trier (1994) should be consulted.

The intestine may be considered as a complex biological membrane, composed essentially of mucosal epithelial cells, tight junctions and intercellular spaces, the glycocalyx at the luminal face of the epithelial apical membrane, and the unstirred layer of water and mucus immediately adjacent to the luminal face of the glycocalyx. Permeation of drug molecules, therefore, requires the passage across these barriers.

1.3.1 Unstirred water layer

As a solute passes from the bulk phase in the intestinal lumen into the cytosol of the enterocyte (or through the tight junctions of the cells), it first encounters, and must pass through, the unstirred water layer (UWL). In humans, the UWL is approximately 500μm in thickness (Thomson and Dietschy, 1984), and is unaffected by forced convection in the bulk phase. Passage of an uncharged solute across the UWL occurs by simple diffusion and is determined by three factors: (i) effective thickness of the UWL (ii) effective surface area (iii) diffusion coefficient of the substrate (Dietschy and Westergaard, 1975).

$$J = \frac{(C_1 - C_2)\,D.A}{d}$$

where J = flux rate
C_1 = solute conc. at luminal bulk phase
C_2 = solute conc. at aqueous-lipid interface
D = free diffusion coefficient
d = thickness of UWL
A = area being considered

It would be expected that the UWL limits the absorption of lipophilic substrates such as long chain fatty acids, and steroids such as bile acids, cholesterol and steroid hormones. Since present-day drugs administered orally are generally fat soluble, their flux across the UWL will also be affected (Csáky, 1984a).

1.3.2 Mucous layer and glycocalyx

A layer of watery mucus covers the epithelial cells of the entire intestine. This layer is composed of water and electrolytes, together with mucopolysaccharides, proteins and nucleic acids which are bound to the brush border membrane by the glycocalyx (Van Hoogdalem, De Boer and Breimer, 1989). The glycocalyx is made up of glycoproteins and, in humans, is between 100 and 500nm thick over the epithelial cells, but somewhat thinner over the goblet cells (Thomson and Dietschy, 1984). The glycocalyx (or fuzzy coat) is covalently linked to lipids and proteins of the apical cell membrane (Van Hoogdalem, De Boer and Breimer, 1989). However, on the basis of diffusion studies, it has been suggested that the intestinal mucus and glycocalyx do not impair the permeation of solutes significantly, and are not contributing factors to the barrier to diffusion of the UWL (Sallee and Dietschy, 1973; Lukie, Westergaard and Dietschy, 1974). However, the binding of drugs to mucus has been described in several studies (Barry and Braybrooks, 1975; Nimmerfall and Rosenthaler, 1980; Shurgers, De Blaey and Crommelin, 1985; Niibuchi, Aramaki and Tsuchiya, 1986), and this may reduce the extent of drug absorption.

1.3.3 Small intestinal wall

Making up the wall of the small intestine are four distinct layers: mucosa, submucosa, muscularis and serosa, going from the luminal side outwards. These are common characteristics of the gastrointestinal tract from the stomach to the large intestine. In the small intestine, the luminal surface is covered by a layer, one cell thick, of mainly columnar epithelial cells and goblet cells with some endocrine cells; these stand on the basement membrane. This layer of cells presents the most important anatomical barrier against solute absorption.

1.3.3(i) *Apical cell membrane*

The apical membrane of the enterocyte comprises densely packed microvilli, which results in a greater surface area for absorption, and the enterocytes are interconnected by tight junctions. It is, therefore, obvious that for solute absorption to occur, it must be as a result of either transcellular permeation (across the epithelial cell) or paracellular permeation (between the enterocytes via tight junctions into the intercellular space). The apical membrane is thicker than the lateral and basal membranes (10.5nm against 7.5nm) (Carr and Toner, 1984), and consists of a phospholipid bilayer into which polysaccharide and proteins are incorporated. The incorporated proteins may span the entire thickness of the membrane, perhaps representing pores and carrier mechanisms involved in the selective permeability of the membrane, or they may protrude from one surface only, for example, those proteins involved in cellular metabolism (Carr and Toner, 1984). The extent of passive diffusion of molecules across the apical cell membrane depends very much upon their lipid-water partition coefficient (Diamond and Wright, 1969). It follows that molecules with a high hydrophilic nature, for example, certain antibiotics and peptides, will have restricted absorption, due to ionisation. After crossing the brush border, compounds then pass into the cytosolic compartment of the cell. As has already been discussed, a notable barrier to the complete absorption of intact peptide drugs is the presence of cytosolic peptidases (reviewed by Alpers, 1994).

1.3.3(ii) *Basal and lateral cell membranes*

The compounds must then cross the basal and the lateral membrane, 7.5nm in thickness, and enter into the interstitial fluid and intercellular cleft, respectively. The barrier function of the basolateral membrane is probably not as great as that of the apical membrane, partly because the lipid fluidity of the former exceeds that of the latter (Dudeja, Brasitus, Dabiya, Brown, Thomas and Lau, 1987), perhaps because of the lower content of glycosphingolipids (Van Meer, 1988).

1.3.3(iii) *Tight junctions*

The tight junctions (zonulae occludentes) are regions of close contact between the apical ends of the epithelial cells, and act to separate the contents of the intestinal lumen from the lateral intercellular spaces. The tight junctions consist of a network of

strands, with the number of strands determining the tight junction permeability: the fewer the number of strands, the greater the permeability (Van Hoogdalem, De Boer and Breimer, 1989). The tight junctions are not as impervious as the name implies. A study by Frömter and Diamond (1972) demonstrated that a large fraction of salt and water transport across the epithelial wall occurs via the tight junctions and intercellular spaces. However, large molecules are excluded from traversing the epithelium via the paracellular route, under normal circumstances. The tight junctions, therefore, act as a passive selective barrier. The leakiness of tight junctions may be increased by the use of certain adjuvants and hyperosmolar solutions by causing some irreversible or reversible degree of damage or alteration to the membrane structure. Examples of these are salicylates, EDTA, methylxanthines and ionic surface active agents (Kararli, 1989). Experimental studies carried out using a variety of such compounds will be discussed later.

1.3.3(iv) *Basement membrane*

Solutes crossing the gut wall by both the paracellular and transcellular routes will subsequently have to permeate the basement membrane and cross into the lamina propria. In rat jejunum, the basement structure has been found to contain abundant fenestrations of between 0.5 and 5μm in diameter (Komuro, 1985). The basement membrane is not considered to provide a major barrier to drug absorption (Csáky, 1984b).

1.3.3(v) *Capillary wall*

After reaching the lamina propria, the drug molecule enters either the blood or lymphatic circulations, or both. The endothelium of the capillaries is fenestrated, and is not considered to act as a barrier to the passage of molecules with sizes of 3nm or less (Kingham and Loehry, 1976; Kingham, Whorwell and Loehry, 1976). The lymphatic circulation would appear to be less important as a route for solute transport, except perhaps for highly lipophilic compounds. The flow of lymph in the intestine is slower than the flow of blood (1-2ml min^{-1} and 2000ml min^{-1} in humans, respectively), and this probably contributes to the lesser part played by the lymphatics (Kararli, 1989). Drugs absorbed by the lymphatics do, however, by-pass the liver

with a consequent reduction in their metabolism, thereby leading to increased overall blood plasma levels.

1.4 Routes and mechanisms of absorption of intact peptides and proteins

As has been previously stated, there are two potential routes of transport across the intestinal wall: transcellular and paracellular. The paracellular route refers to the passage of molecules through tight junctions between the enterocytes, whereas the transcellular route refers to movement across the apical membrane by carrier mediated transport, endocytosis, or diffusion through the aqueous channels or the lipid parts of the membrane into the cytosol and, thence, through the basolateral membrane into the interstitial fluid.

1.4.1 Paracellular pathway

Evidence in favour of the substantial transport of macromolecules by the paracellular route is limited. Nonetheless, paracellular movement of iodized oil (Lipiodol), contained in polyalkylcyanoacrylate capsules of 100 to 200nm in diameter, was traced after being administered directly into the jejunal lumen of the anaesthetised dog (Aprahamian, Michel, Humbert, Devissaguet and Damgé, 1987). Scanning electron microscopy at different elapsed times demonstrated the presence of intact capsules first in the intestinal lumen close to the mucous layer, then in the intercellular spaces and finally in the lamina propria and blood capillaries.

Preparation of these nanocapsules was undertaken in the presence of 0.5% Pluronic F68, a surfactant reported to cause gastric mucosal damage, at a concentration of approximately 1.0% (Nadai, Kondo, Tatematsu and Sezaki, 1972). However, concern regarding the occurrence of adverse structural changes may be unfounded since a separate study by Ohsumi and colleagues determined that a 10-fold increase in Pluronic F68 concentration (5.0%) induced no discernible morphological or functional change in the intestinal mucosa of the rat, even after exposure over a period of 3 months (Ohsumi, Yonezawa, Aizawa *et al*, 1980).

1.4.2 Transcellular pathway

On the whole, evidence that, in the mature intestine, the transport of at least small amounts of intact macromolecules has occurred by the transcellular route is widespread. Morphocytochemical studies have revealed the adsorption of luminally administered horseradish peroxidase (HRP) (Cornell, Walker and Isselbacher, 1971) and of labelled-insulin (150U, in combination with aprotinin, a proteinase inhibitor, and sodium cholate, a permeation enhancer) (Bendayan, Ziv, Ben-Sasson, Bar-On and Kidron, 1990) onto the apical surface membranes of rat small intestinal enterocytes, prior to detection within vesicular and vacuolar structures within the cytosol. Subsequent appearance of these macromolecules at the basolateral membrane and in the extracellular spaces led the authors to conclude that absorption occurred transcellularly, and by an endocytotic process. The retained bioactivity of insulin was confirmed by an observed drop in blood glucose levels, which reached its lowest level, 30 minutes after administration. From this, Bendayan and colleagues estimated the peak absorption of insulin to be 0.5U, an amount not attributable to structural damage, as demonstrated by the retained structural integrity of the intestinal mucosa observed with morphological examination.

Hence, it would appear that the most likely route for the passage of many macromolecules is via the transcellular route. Further evidence for this comes from the view that increasing the lipophilicity of the protein or peptide will facilitate the absorption of the substance (Lee, 1988), and the fact that microemulsions such as liposomes and mixed micelles can enhance absorption (Patel and Ryman, 1977; Fukui, Murakami, Yoshikawa, Takada and Muranishi, 1987; Schilling and Mitra, 1990). Moreover, a review by O'Hagan (1990) states that, in healthy tissue at least, there is little reliable evidence to suggest that macromolecules use the paracellular route.

However, absorption does appear to be very much related to the concentration of ingested protein (Walker and Isselbacher, 1974), since sufficient amounts of protein must escape degradation within the intestinal lumen in order for uptake to occur. Also, the amount of protein must exceed the lysosomal digestive capacity to allow the protein molecules to be transported out of the enterocyte and into the circulation.

1.4.3 Intestinal location providing the optimal site for absorption of intact macromolecules

Since the cellular morphology and enzymatic make-up of the small intestinal mucosa varies as one moves from proximal to distal regions, studies investigating the sites where macromolecular absorption would be optimal are very useful when developing successful oral delivery formulations.

Experiments carried out using rat intestinal loops, both *in vitro* and *in vivo*, have revealed the ileum as the optimal location for intestinal absorption of macromolecules (Schilling and Mitra, 1990; Michel, Aprahamian, Defontaine, Couvreur and Damgé, 1991; Morishita, Morishita, Takayama, Machida and Nagai, 1993). Ileal administration, *in vitro*, of insulin in doses up to 0.5mg ml^{-1} resulted in the transport of only very small amounts of insulin in its intact form (less than 0.2% of initial dose); however, this was significantly greater than that absorbed by the duodenum (Schilling and Mitra, 1990). The authors reasoned that in these experiments, insulin was restricted by the additional barrier presented by the serosal tissue which would not be the case *in vivo*. They further stated that hydrolysis of insulin is not a contributory factor; the absorption differences are, on the whole, a consequence of morphological differences of the cells or tight junctions.

Michel and co-workers (1991) agreed that the ileum was the optimal site of absorption; however, they showed that the administration of encapsulated insulin (100U kg^{-1}) to other parts of the small intestine, *in vivo*, also evoked a significant reduction in glycaemia (51% of the normal value at the duodenum, 53% at the jejunum, and 65% at the ileum) compared with the effects of non-encapsulated insulin, in streptozotocin-induced diabetic rats. This suggests that, in actual fact, proteolytic degradation is a major factor in the limitation of intact peptide absorption.

1.4.4 Morphology of the distal small intestine - M cells

The increase in macromolecular absorption across the ileum compared to more proximal regions of the small intestine may be due to differences in its morphology, as has been previously stated. Peyer's patches are located in the mucosal wall of the small intestine in mammals, and are particularly rich in the ileum close to the ileo-caecal junction (Owen and Jones, 1974; Owen, 1977). They are covered by a special type of cell, the M cell, or membranous epithelial cell, which extends between

the adjacent enterocytes, forming a membrane separating lymphocytes in the mucosa from the intestinal lumen (Owen and Jones, 1974; Owen, 1977). M cells are significantly different from enterocytes as they possess irregular short and wide microvilli which are fewer in number than in enterocytes (Owen and Jones, 1974; Owen and Nemanic, 1978; Smith and Peacock, 1980). Also, vesicles are particularly abundant in the cytoplasm of M cells, reflecting endocytotic activity, and there appears to be fewer lysosomes and therefore a diminished rate of intracellular protein degradation. It has been shown that the rate of uptake of horseradish peroxidase (HRP) by M cells was greater than that of the enterocytes (Owen, 1977). However, a study by Ducroc and associates (Ducroc, Heyman, Beaufrere, Morgat and Desjeux, 1983) stated that the rate of uptake of HRP by both the M cells and the enterocytes was similar. The rate of uptake by the M cells only appeared to be greater since the degradative activity is lower than in the enterocytes and, therefore, the net macromolecular passage is greater.

1.5 Facilitation of peptide and protein absorption across the gastrointestinal wall

One strategy to facilitate mucosal peptide and protein absorption across the intestinal wall is the co-administration of a peptide or protein with a penetration enhancer. This is usually a low molecular weight compound which increases drug absorption by one of several mechanisms e.g. increasing membrane hydrophilicity or expanding the pathway to solute transport through increased permeability of tight junctions (Lee, 1988). Examples of such enhancers are bile salts, chelating agents, surfactants and fatty acids.

However, the far from complete passage of peptides and proteins across the intestinal wall suggests there is at least one other barrier limiting absorption: this is the enzymatic barrier composed of exo- and endopeptidases (see Section 1.1). The enzymatic barrier is one of the most important of a series of barriers limiting peptide and protein absorption from the GI tract. In order to promote oral absorption of peptides and proteins from the GI tract, the components of the enzymatic barrier must be controlled. This can be achieved to some extent by the modification of protein and peptide structure, the use of protease inhibitors, and using the formulation approach. These strategies will now be reviewed in more detail.

1.5.1 Protease inhibitors and penetration enhancers

In the stomach-perfused rat, *in vivo* studies have shown that intraduodenally-administered tetragastrin (4 amino acid peptide) evoked only minor increases in gastric acid secretion (Jennewein, Waldeck and Konz, 1974). When the pancreatic duct was ligated, however, acid secretion increased 11-fold, an amount not dissimilar to that evoked after administration of tetragastrin to a jejunal or ileal loop. Porto-jugular anastomosis, resulting in by-pass of the liver, was also effective in increasing gastric acid secretion, although to a much lesser extent than in the ligated pancreatic duct preparation (3-fold increase). Taken together, the results of this study provide evidence to suggest the pancreatic proteases have an important role to play in the degradation of tetragastrin, with an additional small but significant part played by the hepatocytes.

Attempts to circumvent degradation of orally-administered peptides as big as insulin (51 amino acids) by co-administration with protease inhibitors have been met with a considerable degree of success, at least in the rat (Danforth and Moore, 1959; Kidron, Bar-On, Berry and Ziv, 1982; Takaori, Burton and Donowitz, 1986; Ziv, Lior and Kidron, 1987; Bendayan *et al*, 1990; Morishita *et al*, 1993). This is surprising since the application of one specific protease inhibitor would not be expected to lead to increased peptide bioavailability due to the continuing actions of the other proteases.

The relative impermeability of the gastrointestinal wall provides a further barrier to the absorption of intact peptides. Indeed, studies have determined that potentiation of permeation may be achieved when peptides are administered in the presence of both protease inhibitors and absorption enhancing agents (Kidron *et al*, 1982; Ziv *et al*, 1987; Bendayan *et al*, 1990). Permeation enhancers differ widely in their chemical composition; that they all have absorption-promoting capabilities appears to be the only common characteristic. Examples of such compounds are surfactants, bile salts and non-steroidal anti-inflammatory drugs. The natural occurrence of bile salts in the intestinal tract has brought about extensive application of these compounds in absorption-promoting studies as potentially safe permeation enhancers. Nonetheless, relatively low concentrations of some bile salts (0.2% sodium deoxycholate) causes considerable mucosal damage (Gaginella, Lewis and Phillips, 1977; Nadai, Kume, Tatematsu and Sezaki, 1975; Nakanishi, Masada and Nadai, 1983), whereas

comparable concentrations of sodium taurocholate or sodium cholate did not (Miyamoto, Tsuji and Yamana, 1983). Of interest, then, is the report in a recent patent application (Cortecs Ltd., 1996), whereby the co-administration of carbonate ions (CO_3^{2-}) or bicarbonate ions (HCO_3^-) with bile salts *in vivo* enhanced the absorption of both insulin and salmon calcitonin (as reflected by a drop in plasma glucose levels, and in plasma Ca^{2+} levels, respectively), without affecting cell viability (as observed *in vitro*). The mechanisms underlying the enhancement differ, depending on whether the applied bile acid is conjugated or not. In the presence of sufficient CO_3^{2-} or HCO_3^- ions, the amount of conjugated bile acid required to increase mucosal permeability of the peptide hormone is reduced and, thus, mucosal permeability can be enhanced without concomitant adverse effects. In the case of unconjugated bile acids, rather than increase the degree of cell permeability, CO_3^{2-} or HCO_3^- ions reduce the toxic effect on cells after exposure to bile salts and, thus, greater concentrations of bile acids may be used to increase cell permeability. Additionally, the buffering effect of CO_3^{2-} or HCO_3^- ions can increase the solubility of the bile acids, so that a higher local concentration of the bile salt will be attained, resulting in greater efficacy in enhancing intestinal permeation to bioactive compounds.

Table 1-2 summarises the outcome of a number of studies investigating the ability of protease inhibitors and permeation enhancers to enhance the absorption of insulin when administered to various regions of the small intestine. In addition to measurement of plasma insulin levels, evaluation of blood glucose levels was also carried out, thereby reflecting the absorption of biologically active insulin, as opposed to partially degraded fractions. The importance of this was demonstrated by Ziv *et al* (1987), who showed that a considerable amount of insulin-degraded fragments were of sufficient size to react with the added antibody during radioimmunoassay (RIA). Consequently, misleading results regarding the absorption of insulin may be reported if dependence was solely on RIA of plasma levels of insulin. Nonetheless, it is well established that intact insulin absorption is promoted in the presence of either protease inhibitors or absorption enhancers, such as bile salts. Furthermore, the effects are additive, with blood glucose levels dropping by as much as 66% of original levels in response to insulin dosage of between 100 and 150U ml^{-1} (*c.f.* maximum of 34% drop in blood glucose levels observed in response to insulin in the presence of either

Table 1-2: Blood glucose changes evoked by intra-intestinal administration of insulin, either alone, in the presence of protease inhibitors, or permeation enhancers, or both.

Author	Species	Location	Dose	Blood glucose levels, as percentage of normal (100%)			
				insulin	insulin/Pr[a]	insulin/Ab[b]	insulin/Pr/Ab[c]
Touitou *et al*, 1980	Rat (*in vivo*)	Jejunum	80U ml^{-1}	100%	-	21%	-
Kidron *et al*, 1982	Rat (*in vivo*)	Ileum	12U ml^{-1}	92%	70%	79%	56%
Ziv *et al*, 1987	Rat (*in vivo*)	Ileum	100U ml^{-1}	100%	66%	66%	34%
Bendayan *et al*, 1990	Rat (*in vivo*)	Ileum	150U	-	-	-	35%

[a] *co-administration of insulin with protease inhibitor (Pr)*

[b] *co-administration of insulin with permeation enhancer (Ab)*

[c] *co-administration of insulin with both protease inhibitor and permeation enhancer (Pr/Ab)*

protease inhibitor or absorption enhancer (Ziv *et al*, 1987; Bendayan *et al*, 1990)). Nevertheless, the actual quantity of insulin absorbed across the rat ileum, *in vivo*, after 30 minutes in the presence of both aprotinin and sodium cholate, was reported to be less than 1.0% of the initial amount (Ziv *et al*, 1987).

Thus, the promotion of absorption of large peptides, such as insulin, is very much dependent on the inhibition of proteolysis as well as enhancement of permeation using various absorption promoters. Nonetheless, establishment of the long-term effects of such permeation enhancers is required before this approach can be considered as a viable means of promoting oral drug delivery. Moreover, the very low oral bioavailability of therapeutic peptides (less than 1.0% of initial dose (Ziv *et al*, 1987)), even in the presence of both protease inhibitors and absorption promoters, suggests that to induce any beneficial effect, the dose administered must be immense.

1.5.2 Formulation approach

The formulation approach is another strategy designed to protect the peptide from contact with primarily luminal proteases, and to release the peptide only upon reaching an area favourable to its absorption. Several formulations have been devised eg. water-in-oil-in-water emulsions, liposomes, nanoparticles and soft gelatin capsules with polyacrylic polymers with pH dependent properties.

1.5.2(i) *Liposomes*

Liposomes are vesicles composed of a bilayer containing phospholipids and cholesterol, surrounding an aqueous compartment. In rats, oral administration of liposomally-encapsulated insulin caused a very much smaller hypoglycaemic effect compared with the same dose of intra-peritoneal insulin, delivered free or encapsulated (Patel and Ryman, 1976). Intra-luminal co-administration of encapsulated insulin and a protease inhibitor, however, did result in a significantly decreased plasma glucose level (Patel and Ryman, 1977). In the dog, liposomal insulin was demonstrated to have been transported across the duodenal wall but a subsequent fall in plasma glucose was absent. It was concluded that no more than 1.0% of insulin had reached the circulation in an active form (Patel, Stevenson, Parsons and Ryman, 1982). Thus, it appears that although liposomes enhance absorption, they do not protect against proteolytic degradation.

1.5.2(ii) *Water-in-oil-in-water emulsions*

Water-in-oil-in-water (W/O/W) emulsions have been used as a means of facilitating the gastrointestinal absorption of normally non-absorbed water-soluble polymers. Oil droplets containing their own dispersed phase of fine water droplets are suspended in an aqueous phase; contained within the internal aqueous phase is the therapeutic agent. Equivocal results have been reported as regards the effectiveness of W/O/W emulsions in promoting absorption of peptides, although it was suggested that insulin-containing emulsions may provide a possible means of protecting the peptide from proteolytic degradation and of facilitating absorption (Shichiri, Shimizu, Yoshida, Kawamori, Fukuchi, Shigeta and Abe, 1974). Indeed, Engel, Riggi and Fahrenbach (1968) previously demonstrated marked hypoglycaemia in the rat and gerbil, in response to intraduodenal administration of emulsified insulin.

1.5.2(iii) *Nano/microspheres and nano/microcapsules*

The containment of peptides within spherical structures made of various biodegradable polymers such as polyalkylcyanoacrylate or isobutylcyanoacrylate has been used in an attempt to minimise degradation of the peptide by proteolytic enzymes, and to facilitate permeation of the intestinal wall. On the whole, the results have been promising.

Polyalkylcyanoacrylate nanocapsules (100 - 200nm in diameter), loaded with iodized oil, were reported to enhance the rate of absorption of the entrapped substance from the intestinal lumen to the vasculature of the dog, as measured by increased levels of iodine in mesenteric venous plasma (Aprahamian *et al*, 1987). Further work demonstrated that, although relatively large in size compared with the enterocytes, significant amounts of radio-labelled polystyrene microspheres, up to 300nm in diameter, were absorbed after feeding to rats (Jani, Halbert, Langridge and Florence, 1989).

Insulin (100U kg^{-1}) entrapped in isobutylcyanoacrylate nanocapsules remains biologically active after ileal administration, as evidenced by a significant reduction in blood glucose levels of 65% in streptozotocin-induced diabetic rats (Michel *et al*, 1991). Hypoglycaemia was also evoked following intraduodenal or intrajejunal administration of insulin-containing capsules, although to a slightly lesser extent (52% of control levels). The authors suggested that the presence of M cells in the ileum

may have enhanced absorption. In addition, the duration but not the intensity of the glycaemic response was dependent on the amount of nanocapsules administered; the response may last up to 6 or 20 days after a single oral administration of 12.5 or 50U kg^{-1} encapsulated insulin, respectively (Damgé, Michel, Aprahamian and Couvreur, 1988).

Nanoparticles have also been used as mucoadhesive agents, allowing prolonged residence time of drugs in the gastrointestinal tract and, thus, increasing time for absorption of the entrapped therapeutic agent. *In vitro* studies in the rat have produced favourable results (Bridges, Woodley, Duncan and Kopecek, 1988; Pimienta, Lenaerts, Cadieux, Raymond, Juhasz, Simard and Jolicouer, 1990). For example, hydroxypropylmethacrylate (HPMA) nanoparticles have shown a high level of adhesion to rat ileal intestinal mucosa; bonds appeared to develop very quickly since greater than 50% of the initially-administered nanoparticles remained even after immediate perfusion of the ileum (Pimienta *et al*, 1990).

Other formulations have been developed in order to enhance the bioavailability of orally administered peptide drugs. Perhaps one of the most promising results was obtained by Saffran, Kumar, Savariar, Burnham, Williams and Neckers in 1986. Peptide drugs were coated with polymers cross-linked with azo-aromatic groups to form an impervious film in order to protect orally administered drugs from digestion in the stomach and small intestine. When the azopolymer-coated drug reaches the large intestine (ileocecal junction), the azoreductase produced by the indigenous bacteria reduces the azo bonds, breaks the cross-links and degrades the polymer film. Thus, the drug is released into the lumen of the colon for local action or absorption. The ability of the azopolymer coating to protect peptide drugs was demonstrated in rats, with respect to the peptide hormones vasopressin and insulin; however, further clarification is required as to whether the microbial proteases affect peptide integrity, and the extent to which faecal matter interferes with the absorption process.

1.5.3 Chemical Modification

1.5.3(i) *Peptides with improved resistance to proteases and enhanced lipophilic affinity*

In addition to improving enzymatic stability, modification of the amino acid composition of peptides may also increase their lipophilicity, thereby facilitating permeation of the intestinal wall, and consequently minimising contact of the peptide with proteolytic enzymes at the site of absorption (Lee, 1988).

The replacement of naturally occurring L-amino acids of a peptide for unnatural amino acids (D-configurations) has been employed as a means of improving enzymatic stability. Table 1-3 illustrates this, by comparing the oral bioavailability of dipeptides with varying L- and D-configuration amino acid residues in rats, based on excretion of the administered dose (0.5mmol kg^{-1} body weight) in urine over 240 minutes (Asatoor, Chadha, Milne and Prosser, 1973). Positive correlation existed between the rate of absorption and the rate of hydrolysis.

Table 1-3: Oral bioavailability of dipeptides in the rat

Peptide	Isomer	Excretion (mmol mg^{-1} of creatinine)
Ala-Phe	LL	0.00
	LD	0.00
	DL	0.11
	DD	35.00
Leu-Leu	LL	0.00
	LD	0.32
	DL	0.25
	DD	12.50
Gly-Trp	LL	0.00
	LD	17.0

(Ala, alanine; Phe, phenylalanine; Leu, leucine; Gly, glycine; Trp, tryptophan)

Chemical modification of arginine vasopressin (AVP), a nonapeptide, produced two analogues, 1-deamino-8-D-arginine vasopressin (DDAVP) and 1-deamino-2-tyrosine(o-ethyl)-oxytocin. The intestinal transport of these two analogues were subsequently compared in the rat jejunum, *in vitro* (Vilhardt and Lundin, 1986a).

Detection by radioimmunoassay and quantitative high performance liquid chromatography determined that transport rates across the jejunal wall were greatest for DDAVP, and lowest for AVP, though the level of biological activity was not indicated. Nonetheless, previous reports demonstrated anti-diuresis in response to oral administration of DDAVP in both conscious dogs (Vilhardt and Bie, 1983) and human subjects (Vilhardt and Bie, 1984). This is likely to have occurred as a consequence of both increased resistance to proteases as well as increased lipophilic affinity. Despite this, radioimmunoassay of plasma DDAVP, after peroral administration to human volunteers, established that only between 0.7 and 1.0% of the initial dose actually appeared in the blood (Vilhardt and Lundin, 1986b).

1.5.3(ii) *Carrier-mediated peptide transport*

A more recent approach to the enhancement of oral bioavailability of therapeutic peptides has been the utilisation of carrier-mediated transport mechanisms. As has previously been discussed, poor permeation of the gastrointestinal wall is a major limitation to the successful oral administration of drugs. The means of overcoming this problem is by the use of absorption enhancers such as bile salts, chelating agents or surfactants. However, they may also have the undesirable effect of irreversible damage to or alteration of the epithelium. Utilisation of physiological carrier systems present in the gut, for example, the glucose or bile transporter, may therefore be a viable prospect in promoting oral drug delivery. Success in this field requires modification of the required peptide such that affinity with the carrier system is maintained or improved. Accordingly, a more extensive review is provided in the following sections.

1.6 Facilitation of intestinal absorption by utilisation of carrier-mediated transport systems

1.6.1 Characteristics of carrier-mediated transport

Carrier-mediated transport involves the combination of the transported molecule with a specific site on the transport protein located in the cell membrane, and has a number of characteristic features, which are briefly summarised.

- Substrates transported by carrier-mediated transport are transported at a much more rapid rate than molecules of a similar molecular weight and lipophilic affinity, which cross the membrane by simple diffusion.

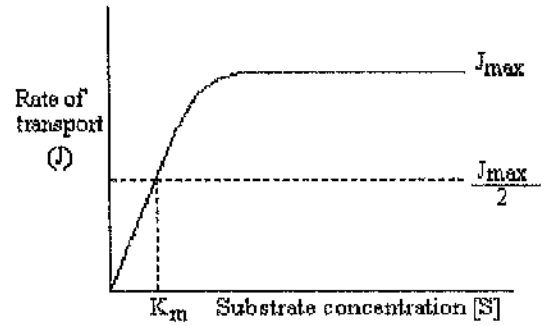


Fig. 1-1:Graphical representation of saturation kinetics exhibited by carrier-mediated systems. (Jmax, maximal transport rate of substrate; K_m, concentration of the transported substrate required for the transport rate to be half-maximal (Jmax/2).

- Since the number of binding sites is finite, saturation kinetics are demonstrated. As the concentration of the substrate is increased, the rate of transport increases until all the available sites are occupied, above which the transport rate increases no further (Figure 1-1). The rate of mediated-transport may be represented by the Michaelis-Menten equation:

$$J = \frac{J_{max}[S]}{K_m + [S]}$$

where J is the rate of transport

J_{max} is the maximal rate of transport

[S] is the concentration of the transported substrate in the compartment from which it is being removed

K_m is the substrate concentration required for half-maximal transport

- Carrier-mediated systems show chemical specificity, so that only molecules with the requisite chemical structure are transported, although specificity is not usually absolute, since a particular transport system may be used by more than one substrate.
- Structurally related molecules may compete for transport. The amount of one substrate transported depends on its concentration relative to that of other substrates, in addition to the affinity of the binding site for the substrates present.
- Transport of a substrate may be inhibited by structurally unrelated compounds that bind to the transport protein and decrease the affinity of the system for the normal transport substrate. Carrier proteins, dependent on metabolic processes, are affected by metabolic inhibitors.

1.6.2 Intestinal glucose transport system

The active uptake of glucose and galactose by carriers located in the membrane of the epithelial cells of the gastrointestinal tract has been well documented (Stern and Jensen, 1966).

The glucose-carrier mediated transport system has been implicated in the transport of glucose- and galactose-conjugated compounds (*β*-naphthyl- and *p*-nitrophenyl β-D-glucopyranoside, and *β*-naphthyl- and *p*-nitrophenyl β-D-galactopyranoside, respectively) in the rat everted jejunal sac (Mizuma, Ohta, Hayashi and Awazu, 1992). Evidence for this was presented by a significant diminution of the absorption clearance of these compounds in the presence of phloridzin, an inhibitor of the glucose transporter. Similarly, substitution of Na^+ ions by K^+ ions in the incubation medium, producing a Na^+ free medium, also showed marked attenuation of the absorption clearance in all cases. Despite this, it was subsequently demonstrated that approximately one-quarter of absorbed *p*-nitrophenyl β-D-galactopyranoside actually appeared on the serosal side as *p*-nitrophenyl glucuronide, which is evidence of a degree of hydrolysis of the substrate during absorption (Mizuma, Ohta, Hayashi and Awazu, 1993). The methodology behind these studies is, however, questionable, since replacement of Na^+ ions with K^+ ions to produce an increase in extracellular concentration of K^+ to approximately 150mM, would result in the creation of conditions outside the normal physiological state.

More recently, the potential for the glucose transport system to improve intestinal absorption of poorly absorbable drugs was investigated (Mizuma, Sakai and Awazu, 1994). The coupling of mono- and disaccharides with a model tripeptide, tyrosylglycylglycine (TGG) markedly improved mucosal to serosal transport of this aminopeptidase-degradable peptide, in the rat everted jejunal sac, with little evidence of hydrolysis of the peptide. Both Na^+-dependent and Na^+-independent mechanisms were involved, indicating that, in addition to facilitating carrier-mediated transport of sugar-coupled TGG, susceptibility to proteolytic hydrolysis appears to be reduced. The applicability of such an approach *in vivo* using larger peptides requires elucidation, but preliminary results are encouraging.

1.6.3 Intestinal bile acid transport system

The approach taken in this study, with a view to enhancing the intestinal absorption of therapeutic peptides, was by way of exploitation of the bile acid transporters present in the plasma membrane of ileal enterocytes. A brief summary of the physiology of bile acid absorption and the enterohepatic circulation will first be presented, prior to a discussion of the current literature involving the use of bile acid transporters for intestinal drug delivery.

1.6.3(i) *Chemical structure of bile acids and bile salts*

Evaluation of the current literature has been complicated by the fact that little distinction has been made between the terms "bile acid" and "bile salt"; generally, these terms have been used interchangeably. Strictly speaking, unionised forms should be referred to as bile acids, whereas ionised forms should be called bile salts, and as far as has been possible, this terminology has been adhered to. Further confusion arises when no clear distinction is made between unconjugated and (taurine- or glycine-) conjugated forms.

Bile acids are produced as the end product of the metabolism of cholesterol by the liver, in which the cholesterol molecule is further modified by the insertion of one or two hydroxyl groups at C-7 and/or C-12, in addition to the one present at position C-3, commonly in the α-configuration (Whiting, 1986). Further modification of cholesterol to produce the bile acids is the saturation of the double bond between C-5

and C-6, the oxidative removal of C-25, C-26 and C-27 from the side chain, and the oxidation of C-24 to a carboxyl group. Bile acids which are synthesised from cholesterol in the liver are termed primary bile acids. These undergo further conversion to secondary bile acids by the action of anaerobic bacteria in the distal intestine (see Section 1.6.3(ii)). In both man and the rat, cholic acid and chenodeoxycholic acid are the two most frequently encountered primary bile acids (Weiner and Lack, 1967). The structures of cholesterol, cholic acid and chenodeoxycholic acid are illustrated in Figure 1-2.

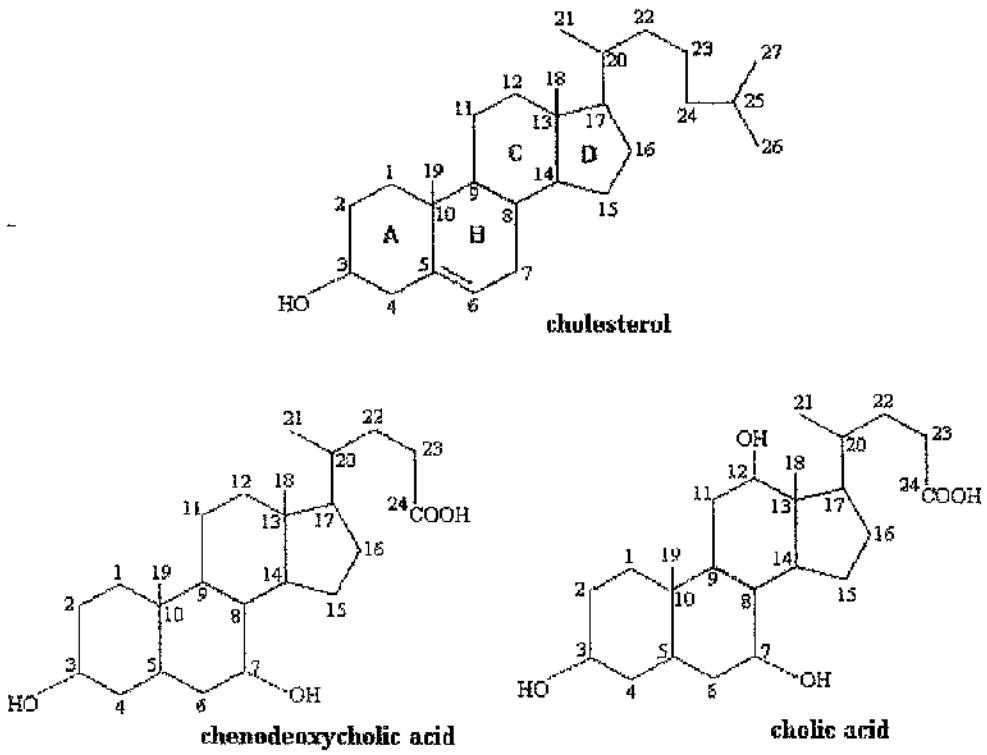
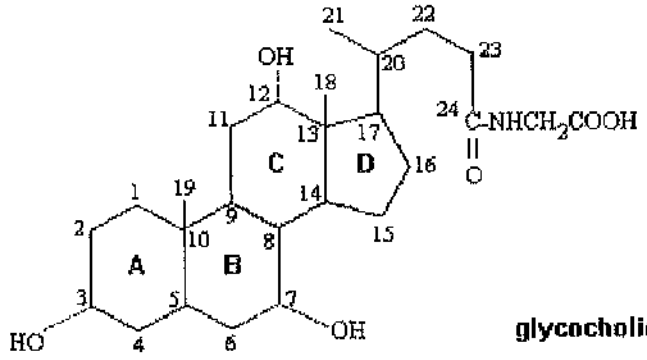


Fig.1-2: Chemical structures of cholesterol, chenodeoxycholic acid, and cholic acid. The carbon atoms are numbered, and the carbon rings of the steroid nucleus are referred to as A, B, C and D.

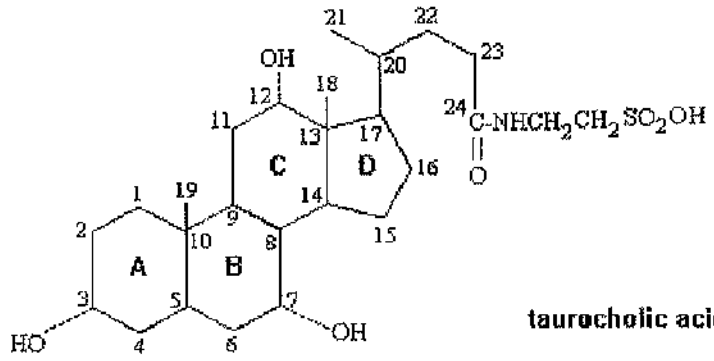
Prior to secretion into the bile, bile acids are conjugated through a peptide linkage at C-24 to either glycine or taurine. In the rat, the relative amounts of glycine- and taurine-conjugated bile acids depends predominantly on the availability of these compounds, and their relative affinities for the coenzyme A derivatives which are involved in the conjugation (Weiner and Lack, 1967). The structures of glycocholic

and taurocholic acid, formed as a result of conjugation of glycine and taurine, respectively, to cholic acid, are shown in Figure 1-3.

Consequently, the pKa of the bile acids and bile salts will depend on the unconjugated or conjugated state of the bile acid/salt. For example, unconjugated bile acids have a pKa of approximately 6.0, and thus, the ratio of salt:acid will vary depending on the



glycocholic acid



taurocholic acid

Fig. 1-3: Chemical structures of glycocholic acid and taurocholic acid. The carbon atoms are numbered 1 to 24, and the carbon rings of the steroid nucleus are referred to as A, B, C and D.

pH of the medium. On the other hand, the pKa values for glycine and taurine conjugates are lower, at approximately 4.0 and 2.0, respectively, and as such, at physiological pH, they always occur as salts (Dietschy, 1968). The conjugation of bile acids with glycine or taurine is of functional relevance since not only does it prevent precipitation in the presence of acid or divalent cations (Whiting, 1986), but it also increases the active absorption of bile salts in the terminal ileum, since conjugated bile salts have a higher affinity for the ileal bile salt transporter than unconjugated bile acids (Gordon, Miner and Kern, 1971; Aldini, Roda, Lenzi, Ussia *et al*, 1992).

Furthermore, conjugation reduces passive transport in the jejunum and proximal ileum, since the ionised state reduces lipophilic affinity, thereby making bile salts more available to participate in lipid digestion at these locations (see Section 1.6.3(iii)).

1.6.3(ii) *The enterohepatic circulation*

This exists as a highly efficient mechanism for the conservation and reutilisation of the bile salt pool. It has been estimated that in man, the liver secretes approximately 20 to 30g bile salts daily, and of this, only about 0.8g appears in the faeces as bile salt excretory products per day (Bergström, 1959). Bile salts are synthesised and excreted by the hepatocytes of the liver into the bile canaliculi and are stored, and subsequently concentrated, in the gall bladder in the periods between meals. Contraction of the gall bladder, for example, in response to cholecystokinin (CCK), released by the duodenal mucosa in response to the presence of lipids in the duodenum, results in emptying of bile salts from the gall bladder into the duodenum (Ivy and Oldberg, 1928).

Bile salts are conserved through reabsorption across the small intestinal wall, and return to the liver primarily via the hepatic portal circulation. However, a small proportion of bile salts enter the colon; here, primary bile salts undergo conversion to secondary bile salts by anaerobic bacteria. Taurocholate and glycocholate are deconjugated and dehydroxylated to deoxycholic acid, and chenodeoxycholic acid becomes lithocholic acid. Up to one-half of deoxycholic acid is reabsorbed and taken up by the liver for reconjugation with glycine or taurine, whereas lithocholic acid is relatively insoluble and thus poorly absorbed (Whiting, 1986). A more detailed account of the processes involved in the enterohepatic circulation now follows.

1.6.3(iii) *Physiology of bile salt absorption*

It was first established in 1878, by Tappeiner, that various sites of the small intestine differed in their ability to reabsorb bile salts, with bile salt reuptake being more rapid in the ileum than in more proximal regions. Confirmation of this came from the work of Fröhlicher (1936), nearly 60 years later. Nevertheless, it was not until almost a century after Tappeiner's first claims that substantial progress in this area was made. Equivocal evidence for the importance of the distal small intestine in the maintenance of the enterohepatic circulation in rat, guinea pig, hamster and human was reported

(Baker and Searle, 1960; Borgström, Lundh and Hofmann, 1963; Lack and Weiner, 1961; Playoust and Isselbacher, 1964; Holt, 1964). It was found that as one descended the rat small intestine, *in vivo*, the rate of sodium glycocholate and sodium taurocholate reabsorption increased in an exponential manner with distance (Baker and Searle, 1960). So, although absorption was evident over the entire length of the small intestine, there was indirect evidence that a specific system may exist in the mucosal epithelium of the ileum for the absorption of these bile salts.

Elucidation of this concept was first provided by Lack and Weiner (1961), who determined the transport mechanisms of the bile salts, taurocholate and glycocholate, in the everted gut sacs of the rat and guinea pig. Initially, the concentrations of the bile salt under consideration were equal in both the serosal and mucosal compartments. After a 90 minute incubation period, only those intestinal sacs prepared from the distal quarter of the small intestine showed a mean serosal:mucosal ratio of much greater than 1.0. Thus, only the ileum appeared capable of transporting bile salts against a concentration gradient. Also, since the transmural potential difference of these sacs was, at most, only a few millivolts (Clarkson, Cross and Toole, 1961), transport could not be ascribed to a favourable electric gradient. Evidence for the presence of an active transport system in this region was further provided by inhibition of oxidative metabolism by replacement of the oxygen used to aerate the bathing medium, or addition of the oxidative metabolic inhibitors, 2,4 dinitrophenol or sodium azide, resulting in the marked attenuation of bile salt transport. Finally, the transport activity of the distal segment reached a maximum with increasing concentrations of either glycocholate and taurocholate, indicative of a saturable process (see Section 1.6.1).

Subsequent experiments produced similar results in the hamster (Playoust and Isselbacher, 1964) and the rat (Playoust and Isselbacher, 1964; Holt, 1964), in that the ileal transport of bile salts against a concentration gradient was shown to obey Michaelis-Menten (saturation) kinetics. Further, the dependence of the transport system on Na^+ ions was confirmed in both these studies by the marked inhibition of reuptake when Na^+ ions were omitted from the incubation medium. Additionally, competitive inhibition of taurocholate uptake by the additional presence of cholate or glycocholate was evident, suggesting the existence of a common receptor site for the active transport of these bile salts in the ileum (Playoust and Isselbacher, 1964). Lack

and Weiner (1966) expanded further on this evidence by showing that some synthetic bile salts, as well as naturally occurring bile salts, were able to utilise the bile salt carrier protein, in the guinea pig everted ileal sac. The presence of a negative charge on the bile salt side chain was a prerequisite. Nevertheless, because these experiments were carried out *in vitro*, and because no K_m values (see Section 1.6.1) for the different bile salts were determined (serosal:mucosal ratios were typically measured at only one concentration), the results obtained should be considered with caution.

More recently, insight into the different mechanisms of the small intestine involved in the maintenance of the enterohepatic circulation has been provided. It is now well established that active transport and passive diffusion of bile salts exists from the ileum and jejunum, respectively, of several mammals such as the pig (Juste, Legrand-Defretin, Corring and Rerat, 1988), the rat (Yokote, 1994; Dakka, Dumoulin, Chayvialle and Cuber, 1995), the guinea pig (Amelsberg, Schteingart, Ton-Nu and Hofmann, 1996), and the rabbit (Aldini, Montagnani, Roda, Hrelia, Biagi and Roda, 1996), as well as the fish (Honkanen and Patton, 1987). Passive diffusion of bile salts from the jejunum of turkeys was also demonstrated (Sklan, 1980). More specifically, it was concluded that, in the rabbit, conjugated trihydroxy bile salts are actively absorbed from the ileum, conjugated dihydroxy bile salts share both active and passive ileal absorption, and unconjugated dihydroxy bile acids diffuse passively across the entire length of the small intestine, according to their lipophilic affinity (Aldini *et al*, 1996). Additionally, Amelsberg and colleagues (1996) proposed that the reuptake of conjugated bile acids occurs by at least two mechanisms: carrier-mediated transport of glycine and taurine-conjugates, and passive absorption in the protonated (uncharged) form of glycine conjugates. The latter, they suggest, may occur in the presence of the jejunal acidic microclimate, which exists close to the apical membrane of the jejunal enterocyte. In humans and rats, an acidic microclimate of between pH 5.4 and 6.2 was measured with H^+ ion sensitive microelectrodes (Lucas, Schneider, Haberich and Blair, 1975; Lucas, Blair, Cooper and Cooke, 1976; Lucas, 1983; M°Ewan, Daniel, Fett, Burgess and Lucas, 1988). However, at the lowest measured pH, only a very small fraction of glycine conjugates (~ 7%) would be in the protonated form, although in some pathological states, it is conceivable that passive absorption of protonated glycine conjugates plays an important role in bile acid/salt absorption.

The presence of an active transport system for intestinal bile salt absorption, present only in the ileum, has thus led to the view that the distal small intestine is the major site of bile salt absorption within the enterohepatic circulation. However, M°Clintock and Shiau (1983) suggested that this idea has arisen due to predominantly *in vitro* studies in which experimental concentrations of bile salts were below physiological levels (luminal bile salts in the fed rat are of the range: 10 to 12mM in the proximal jejunum; 18 to 20mM in the mid-intestine; 2-3mM in the terminal ileum (Dietschy, 1968)). They showed that in the rat, *in vitro*, the uptake of low concentrations of taurocholate (0.5-1.0mM) from the jejunum was significantly less compared with that from the ileum. This is consistent with the earlier *in vitro* experiments. By contrast, jejunal and ileal uptake of taurocholate at concentrations above critical micellar concentration (4.0-15.0mM, depending upon the type of bile salt) was equal and linear with respect to concentration. However, in anaesthetised rats, approximately 30% of luminally administered taurocholate was absorbed from the proximal jejunum over a 90 minute period, yet only approximately 1.0% was absorbed from more distal small intestinal regions. Furthermore, in the conscious fed animal, 60% of administered taurocholate was recovered in the bile before the bolus reached the distal ileum. The authors proposed that, because the luminal concentration of taurocholate is highest proximally, predominantly passive absorption contributes significantly to the maintenance of the enterohepatic circulation. Ileal active transport appeared to be most efficient at low concentrations, and may act to absorb the remaining taurocholate from the distal small intestine. This conflicts with the premise that, since intestinal transport of bile salts is dependent on both the luminal pH and the pKa of the bile salt, taurine-conjugated bile salts (pKa~2) would not be expected to cross the small intestinal wall rapidly by simple diffusion of the non-ionised form.

Further evidence for the re-evaluation of the role of the proximal small intestine in bile salt absorption comes from Juste *et al* (1988). In pigs, *in vivo*, the rate of bile salt reabsorption in the distal ileum and large intestine was only 20% that of the rate for the whole intestine (small and large intestine), implicating a sizeable contribution by the proximal intestine in the maintenance of the enterohepatic circulation.

Finally, it should also be recognised that a number of investigators have demonstrated jejunal expression of bile salt carrier proteins in both rats and humans following

ileostomy, which presumably occurs as a consequence of jejunal cellular adaptation to transport requirements (review by Swaan, Szoka and Øie, 1996).

1.6.3(iv) *Ileal transcellular transport of bile salts - a proposed model*

The general consensus is that bile salts are transported across the ileal enterocytes by way of an active, carrier-mediated, Na^+ dependent, co-transport mechanism.

A model for the transcellular transport of bile salts in rat ileal enterocytes was proposed by Lin, Mullady and Wilson (1993), based on studies investigating the cloning and expression of the ileal bile salt carrier. The transport of bile salts across the brush border membrane is by a 99-kDa protein which constitutes a Na^+ dependent active transport system. Within the cytosol of the enterocytes, bile salts bind either to actin (43-kDa protein) or 14- and 35-kDa cytosolic proteins, and are transported either to a microsomal 20-kDa protein, or directly to the basolateral membrane. Bile salts may then be transferred to the basolateral membrane associated protein (59-kDa) before their egress from the cell via the 54-kDa integral basolateral membrane protein and an unidentified anion exchange mechanism (Figure 1-4).

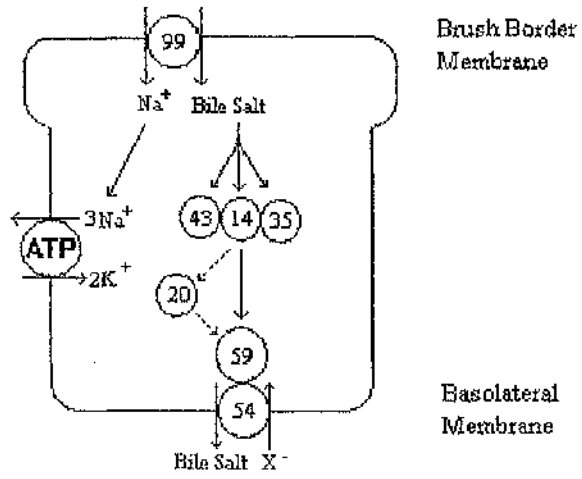


Fig. 1-4: Mechanism of ileal transcellular transport of conjugated bile salts as proposed by Lin and associates (1993). Transport of bile salts at the ileal brush-border membrane occurs by way of a Na^+-dependent active co-transporter (99 k-Da). The bile salts are translocated through the cell by cytosolic proteins (14, 35 and 43 k-Da) to the basolateral membrane associated protein (59 k-Da),and exit the cell via a basolateral membrane bile salt/anion (X) counter-transporter (54 k-Da). An indirect pathway involving a microsomal protein (20 k-Da) (dashed arrows) may also contribute.

1.6.3(v) *Hepatic uptake of bile salts and bile acids - a proposed model*

The review by Swaan *et al* (1996) has outlined the most recent findings regarding the mechanisms behind the uptake of bile salts and acids from the sinusoidal blood supply and the excretion into the canaliculi of the liver. Two separate transport steps are involved: the uptake of bile salts and bile acids across the sinusoid-facing membrane of the hepatocyte, and transport of bile salts/acids across the canalicular membrane (Fig. 1-5).

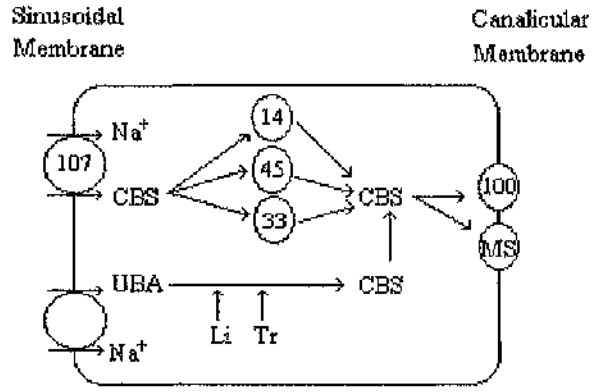


Fig. 1-5: Proposed model for the hepatic uptake of bile acids and bile salts, as reviewed by Swaan et al (1996). Conjugated bile salts (CBS) are transported at the sinusoid-facing membrane by a Na^+-dependent active symporter (107-kDa), and are translocated intracellularly by cytosolic proteins (14-kDa, 33-kDa and 45-kDa). At the sinusoid-facing membrane, unconjugated bile acids (UBA) are uptaken by a separate Na^+-dependent transporter, and undergo conjugation to glycine or taurine within the cell by interaction of the enzymes, ligase (Li) and transferase (Tr). Conjugated bile salts are then exported across the canalicular membrane via a specific bile salt transporter (100-kDa) and a multi-specific organic anion transporter (MS).

Transport of conjugated bile salts across the sinusoid-facing membrane is thought to occur via a Na^+-dependent carrier mediated symporter (107-kDa), and transport of unconjugated bile acids was suggested to occur via a different Na^+-dependent carrier (Swaan *et al*, 1996). The conjugated bile acids undergo translocation through the cell to the canalicular membrane via the smooth endoplasmic reticulum (SER) and cytosolic bile salt-binding proteins, of which three have been identified in rat and human: glutathione S-transferase (45-50 kDa); Y' bile salt binder (33-kDa); fatty acid-binding protein (14-kDa). Unconjugated bile acids are conjugated with glycine or taurine in the cytosol by interaction of a ligase and a transferase, prior to transport

from the cell. Two separate transporters are involved in the export of bile salts across the canalicular membrane. Monovalent bile salts are carried by a specific bile salt transporter (100-kDa), whilst dihydroxy bile salts are transported by a multi-specific organic anion carrier.

Moreover, a comparison of cDNA coding regions and protein amino acid sequences of ileal and liver bile salt transporters in human, rat and hamster has revealed that, although similar regions in the carrier polypeptide chains exist, the hepatic and ileal bile salt transporters are not identical (Swaan *et al*, 1996).

1.6.3(vi) *Use of the ileal bile salt transporter for enhancing intestinal drug delivery*

Structural modifications of bile salts by coupling to poorly absorbable drugs may enable the enhancement of intestinal absorption of such molecules, since the bile salt is an endogenous chemical structure, recognised by the bile salt active transporter in the ileum. The initial interaction between the bile salt and the ileal transporter is reported to take place within a hydrophobic space of the enterocyte membrane, and involves three steps (Lack, 1979; Lack, Tantawi, Halevy and Rockett, 1984):

(1) interaction of a recognition site on the carrier protein with the steroid nucleus of the bile salt.
(2) an electrostatic interaction between the negative side chain of the bile salt and a cationic site of the carrier protein.
(3) an interaction of Na^+ with an anionic site on the carrier protein, located in close proximity to the cationic site of the carrier protein.

An account of the necessary characteristics required of modified bile salts in order to accomplish active uptake has been produced by Lack (1979); moreover, experimental confirmation of these criteria has been reported (reviewed by Kramer and Wess, 1996).

They are as listed:

(1) at least one axial hydroxyl group (that is, -OH group extended in the direction perpendicular to the axis of steroid rings) at position C-3, C-7 or C-12 of the steroid nucleus.

(2) a *cis* configuration of rings A and B of the steroid nucleus to ensure hydrophobic interaction with the transporter (see Figure 1-3 for position of A and B carbon rings).

(3) retention of an anionic side chain at position C-17 on the D-ring of the bile salt molecule required for electrostatic interaction with the cationic site of the transporter. Uptake of a bile salt/acid with a positively charged or neutral side chain is markedly attenuated.

The retention of the characteristics of naturally occurring bile salts when producing a drug-bile acid conjugate, as far as is possible, appears to be essential for successful targeting of such a system for drug delivery.

Ho (1987) carried out liver absorption and biliary excretion studies *in situ*, as well as intestinal perfusion experiments of the jejunum and ileum of the rat, and determined that 3-tosyl-cholate and 3-benzoyl-cholate, and 3-iodo-7α,12α-dihydroxycholan-24-oate were dealt with in the same way as naturally occurring bile salts. However, Kramer and Wess (1996) suggested that this was not unexpected since the compounds investigated were bile salt analogues with minor structural modifications, rather than drug-bile acid conjugates.

It has been reported that the Caco-2 cell line expresses the ileal bile transporter, and is able to transport bile acids unidirectionally from the apical to the basolateral membrane (Hidalgo and Borchardt, 1990). Coupling of cholic acid or taurocholic acid, at position C-3, with renin-inhibitory peptides (RIPs) resulted in a high affinity with the Caco-2 cell bile salt transporter, although bioavailability remained very low (Kim, Harrison, Ruwart, Wilkinson, Fisher, Hidalgo and Borchardt, 1993). Comparable results were also demonstrated in the perfused rat ileum, in that the RIP-cholic acid conjugate went undetected in blood samples taken from the mesenteric vein (Kim *et al*, 1993). This would seem to indicate that either the translocation of the conjugate through the cell or its efflux from the cell was impeded in some way.

Recently, extensive work carried out by Kramer *et al* (1994), investigated the intestinal absorption of model peptides coupled to the 3-position of modified 3β-(ω-aminoalkoxy)-7α,12α-dihydroxy-5β-cholan-24-oic acid in the anaesthetised rat. Coupling to the 3-position was deemed necessary since a previous study had

demonstrated that drug-bile acid conjugates with attachment of the drug at this position were recognised as natural bile salts by the bile salt transport systems of the hepatocytes of the liver (Kramer *et al*, 1992; Wess *et al*, 1992). Furthermore, additional studies have shown that a negative charge in the side chain attached to C-17 of the bile salt molecule, and at least one hydroxyl group in positions 3, 7 or 12 of the steroid nucleus are essential for optimal recognition of a bile salt molecule by the bile salt carrier systems in the liver and the ileum (Lack and Weiner, 1967; Anwer, O'Máille, Hofmann, Dipietro and Michelotti, 1985; Hardison, Heasley and Shellhamer, 1991; Kramer *et al*, 1993).

The model peptides constructed by Kramer and associates (1994) were composed of D-alanine and D-proline, and had 1, 2, 4, 8 or 10 amino acid residues (naturally occurring L-amino acids were replaced with D-amino acid residues to prevent enzymatic hydrolysis of the peptide backbone; see Section 1.5.3(i)), so that when conjugated with the bile acid at C-3, they created side chains of 1, 2, 4, 8 or 10 amino acid residues. The interaction between the peptide-bile acid conjugates and the ileal bile salt co-transport system was demonstrated by measuring competitive inhibition exerted by the conjugate on [^{3}H] taurocholate uptake by rabbit ileal brush border membrane vesicles. Peptide-cholic acid conjugates with up to four amino acid residues in the peptide side chain were able to inhibit the uptake of [^{3}H] taurocholate in a dose-dependent manner. Coupling of eight amino acid residues, however, resulted in a significant drop in the affinity for the ileal bile salt transporter, yet it remains unclear as to what the critical number of amino acid residues is as to cause this loss in affinity.

This study (Kramer *et al*, 1994) was taken a step further by investigating the ileal absorption of a tetrapeptide-bile acid conjugate, S3744, modified with an additional fluorescent labelling group in the rat, *in vivo*. The S3744 conjugate was found to be actively transported by the ileal bile salt transporter, while neither the parent peptide (S1037) nor its *t*-butylester (S4404) appeared in bile. The transport profile of S3744 into bile was comparable to that of a natural bile salt. Additionally, competitive inhibition of uptake of ^{3}[H]taurocholate provided further evidence of the involvement of carrier-mediated transport.

The results of this extensive study demonstrated that targeting the intestinal bile salt carrier is a possible approach to facilitate the uptake of otherwise poorly absorbable

molecules. The review by Kramer and Wess (1996) suggested that for successful absorption of the drug-bile acid conjugate, the side chain at C-17 should remain untouched, and the drug moiety should be attached to the steroid nucleus, preferably at C-3. In addition, since the intact peptide-bile acid conjugate was excreted by the liver into bile (Kramer *et al*, 1994), it would appear that successful oral delivery of the intact peptide into the systemic circulation depends, at least in part, on the release of the peptide before it reaches the liver, perhaps by hydrolysis of the bond between the bile salt and the peptide, or by diminished affinity of the analogue for the hepatic bile salt transporter.

1.7 Objectives of the present study

The encouraging outcome of the study by Kramer and associates (1994), in which model peptides coupled with cholic acid demonstrated a high affinity for the ileal bile salt transporter with a similar transport profile to natural bile salts, demonstrates the practicability of this approach to the facilitation of peptide absorption. However, although conjugation with bile salts seemed to reduce the barrier to permeation, the work of Kramer *et al* (1994) did not involve the coupling of biologically-active peptides, and as such, there was little indication as to whether the peptide moiety would retain its bioactivity. Indeed, Kim *et al* (1993) reported the very low bioavailabilty of renin-inhibitory peptides coupled with cholic acid, even though a high affinity with the ileal bile acid transporter was demonstrated.

In the present study, we wished to expand on the work of Kramer and colleagues, by coupling of a biologically-active peptide with a bile acid, in an attempt to determine the viability of such a compound in oral drug delivery. While Kramer and Wess (1996) maintained that successful utilisation of the active bile salt carrier may only be achieved if the peptide moiety is attached to the steroid nucleus (preferably at C-3), and the bile acid side chain at C-17 remains unmodified, we were of the view that coupling of the peptide to the bile acid at the site where glycine or taurine naturally attach may be a viable alternative. Cholic acid (3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid) was selected as the bile acid since it has been established that, along with chenodeoxycholic acid, it is the most frequently encountered bile acid in man and the rat (Weiner and Lack, 1967). The peptide to be conjugated with cholic acid needed to be of relatively small size, at least for preliminary experiments, and measurable with

a reliable assay. To this end, we decided upon tetragastrin, a peptide comprising the carboxy-terminal four amino acid residues (tryptophan-methionine-aspartate-phenylalanine amide) of gastrin. Using the stomach-perfused rat model, we were able to assess the ability of our "drug" in permeating the small intestinal wall and entering the systemic circulation by measurement of the increase in gastric acid secretion. A detailed review of the actions of gastrin on gastric acid secretion thus follows.

1.8 Gastric Acid Secretion

1.8.1 Regions of the stomach

The stomach may be divided into four distinct regions: the cardia; the fundus; the body and the antrum (Fig. 1-6), although very often, for convenience of notation, a distinction is only made between the fundus and the antrum.

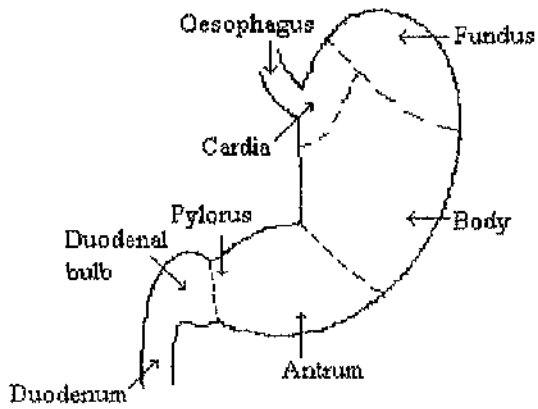


Fig. 1-6: The major anatomical subdivisions of the stomach

The gastric mucosa consists of a columnar epithelium with numerous pits (a recent description is provided by Kutchai, 1998). Opening into these are various types of glands depending on the region of the stomach in which they are located. Within the gastric mucosa of the cardia, mucous secreting cells open into each pit. The glands opening into the pits located in the fundus and body are, however, of a more complex nature (Fig. 1-7). Located here are the gastric glands which, in addition to mucous secreting cells, contain parietal (or oxyntic) cells involved in gastric acid secretion, and chief (or peptic) cells which secrete pepsinogens. Gastric glands are absent from the antrum; however, present in the antral pits are chief cells, and G cells which, in adult mammals, are involved in the release of the gastrointestinal hormone, gastrin. A

general overview of the structure of the gastric mucosa can be found in Lloyd and Debas (1994).

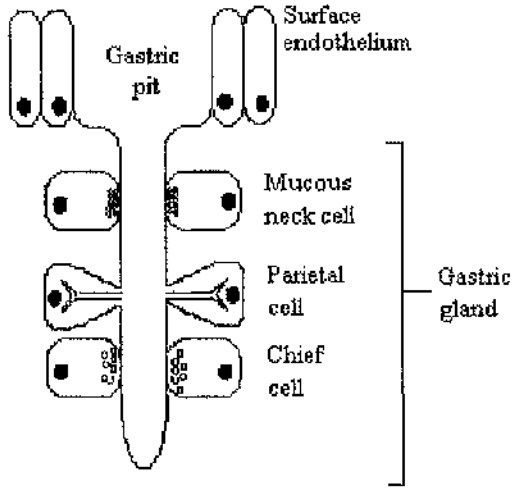


Fig. 1-7: Structure of the gastric gland, present in the fundus and body of the stomach. Solid symbols denote cell nuclei, and small open symbols denote vesicles.

1.8.2 Secretion of gastric acid - morphological changes

As has been previously stated, the parietal cells of the fundus and body of the stomach are involved in gastric acid secretion, the rate of which, in humans, ranges from between 1.0 and 5.0mEq hr^{-1} during basal secretion (Wormsley and Grossman, 1965) to between 6.0 and 40.0mEq hr^{-1} at maximal stimulation (chapter by Kutchai, 1998). Discernible changes in the morphology of parietal cells accompanying gastric acid secretion were first observed by Camillo Golgi in 1895. More recent ultrastructural studies have shown that, at rest, the cell is characterised by numerous mitochondria, a network of tubovesicles, and an internalised intracellular canaliculus (Ito, 1987). Studies using electron microscopic radioautography have demonstrated that the canaliculi, as opposed to secretory granules, are the site of initiation of acid secretion (Sekiguchi, Nagaya, Satoh and Shino, 1992; Scott, Helander, Hersey and Sachs, 1993). Stimulation of the parietal cell results in the fusion of tubovesicles with the plasma membrane, by rearrangement of the cellular cytoskeleton, thereby opening the cell into the lumen of the gland (Ito, 1987). In humans, HCl is secreted into the gastric lumen in concentrations of up to 150mM, equivalent to a million fold concentration of H^+ ions (Nordgren, 1963).

1.8.3 Secretion of gastric acid - cellular mechanisms

The mechanism of acid secretion is well documented, the current view being as follows. The process depends primarily on the presence of an H^+/K^+ ATPase located in the apical membrane of the parietal cell (Rabon and Reuben, 1990), and has an absolute dependence on oxygenation, as reflected by the large mitochondrial content of the cell; glycolysis is unable to support acid secretion (see Sachs (1994) for a review). The carrier protein extrudes H^+ ions from the cytosol of the parietal cell into the canaliculus (and then into the lumen of the gastric gland) in exchange for the entry of K^+ ions. An intracellular build up of K^+ ions does not arise since the mucosal membrane is highly permeable to K^+ ions which permeate back into the lumen. The removal of H^+ ions from the cell by the H^+/K^+ ATPase results in an increase in HCO_3^- ions within the cytosol. In essence, the production of H^+ and HCO_3^- ions arises from the dissociation of carbonic acid (H_2CO_3), formed by combination of CO_2 and H_2O; the reaction is catalysed by carbonic anhydrase (CA) which is closely associated with the canalicular membrane (Cross, 1970). Regulation of intracellular pH is achieved by a Cl^-/HCO_3^- countertransporter located on the basolateral membrane, which removes HCO_3^- ions from the cell. Cl^- ions move into the cell, via the exchanger, down their electrochemical gradient, and subsequently pass into the lumen of the gland via an electrogenic anion channel situated on the apical membrane (general overview by Kutchai, 1998). The movement of Cl^- ions thus preserves electroneutrality while the movement of water subsequent to ion transport preserves isosmolality. The result is a secretion of 150mM HCl solution. Intracellular pH is also maintained by the activity of a second exchange protein, the Na^+/H^+ countertransporter, located on the basolateral membrane which normally ejects H^+ ions in exchange for entry of Na^+ ions. The activity of this exchanger is diminished during stimulated gastric acid secretion. A summary of the main processes involved is illustrated in Figure 1-8.

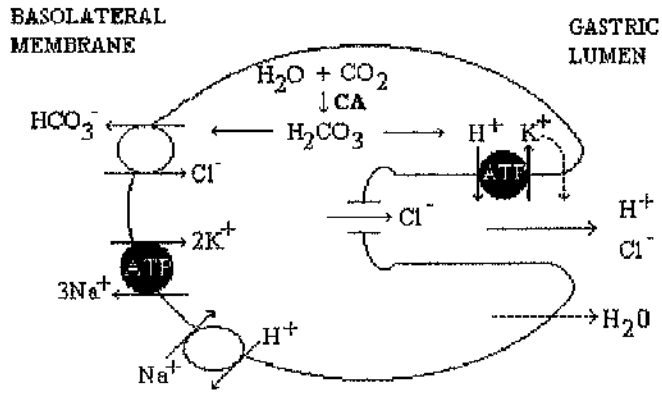


Fig. 1-8: Simplified representation of the major ionic transport processes involved in the secretion of H^+ and Cl^- of the parietal cells of the stomach. (CA denotes the enzyme, carbonic anhydrase; open circles denote facilitated transport; filled circles (labelled ATP) denote energy dependent transport processes.)

1.8.4 Gastric bicarbonate secretion

The existence of a gastric alkaline secretion was documented in the 1950s when Grossman (1959) demonstrated the secretion of HCO_3^- ions from a canine antral pouch, in which parietal cells were absent. Hollander (1963) further described a similar secretion from the denervated fundal pouch of the dog, following instillation of acetylcholine or iodoacetamide. Although the results demonstrated that, on the whole, the concentration of secreted HCO_3^- ions was less than that of interstitial fluid, Hollander suggested that mixing with the parietal acid secretion would result in neutralisation and dilution of this secretion. In 1966, Makhlouf and McManus proposed a two-component model for gastric secretion, in which they referred to gastric acid secretion as the parietal component. In addition, another secretion was described, which had a composition similar to that of interstitial fluid with the exception that K^+ and HCO_3^- ions were higher in concentration. This was thought to arise from the chief cells and the mucous secreting cells of the gastric mucosa, giving rise to the non-parietal component. The rate of HCO_3^- secretion increases in the presence of acid in the gastric lumen (review by Flemström, 1994), such that the maximal rate of secretion is 10% that of maximal HCl secretion (Kutchai, 1998) and, furthermore, studies on the guinea pig stomach have demonstrated potentiation of HCO_3^- secretion by release of acetylcholine from the vagus nerves (Garner and Flemström, 1978).

The release of bicarbonate, along with mucus secretion, is considered to act as a protective buffering mechanism against the potentially harmful effects of acid in the gastric lumen (Garner and Flemström, 1978). However, this is a topic under debate; following studies on canine chief cells in culture (Sanders, Ayalon, Roll and Soll, 1985), Machen and Paradiso (1987) proposed that protection arises predominantly from the low permeability of the apical membrane of epithelial cells to H^+, rather than from buffering by an alkaline mucous barrier.

1.8.5 Control of gastric acid secretion

The three principal effectors of gastric acid secretion are gastrin, histamine and acetylcholine (as reviewed by Wood, 1987). Both gastrin and histamine act directly on specific receptors situated on the parietal cell to stimulate acid secretion but, in addition, studies on the dog (Chuang, Tanner, Chen, Davidson and Soll, 1992) and the rat (Lloyd, Raybould, Taché and Walsh, 1992) have demonstrated that gastrin may also exert its effect indirectly by stimulating the release of histamine. Acetylcholine, released from vagal nerve varicosities, activates a specific receptor on the parietal cell to initiate the extrusion of H^+ ions (described by Lloyd and Debas, 1994). Also, the release of another neurotransmitter, gastrin releasing peptide (GRP), from the vagi results in the stimulation of G cells to release gastrin (review by Bunnett, 1994). GRP, isolated from the stomach and intestine of the pig (McDonald, Jörnvall, Nilsson, Vagne, Ghatei, Bloom and Mutt, 1979), was discovered to have a remarkable similarity to the peptide, bombesin (discovered in extracts prepared from the skin of two European amphibians (genus *Bombina*) in 1970 by Erspamer, Erspamer and Inselvini). Bombesin, a peptide with 14 amino acid residues, shares 9 out of 10 of its carboxy-terminal residues with GRP, which is now regarded as the mammalian counterpart of bombesin (as reviewed by Bunnett, 1994).

The role played by gastrin in the regulation of gastric acid secretion is of most relevance to this project and, thus, will be discussed in more detail.

1.9 Gastrin

The role of gastrin is summarised in most physiology textbooks, and an extensive account has been written by Walsh (1994). Gastrin is a gastrointestinal hormone secreted by the G cells of the antrum of the stomach. It was discovered in 1905 by

Edkins, who proposed that digested food components caused the release of a substance which stimulated gastric acid secretion. However, the actions of gastrin were demonstrated unequivocally by Grossman, Robertson and Ivy only in 1948. They reported increased secretion, in the dog, of gastric acid from a transplanted fundal pouch in response to distension of a denervated antral pouch. So, the possibility of mediation by a neural mechanism was ruled out, and the existence of a circulating stimulant released from the gastric antrum was implicated. The outcome of this experiment, along with the results of a study by Komarov (1938), in which it was determined that a non-histaminergic substance of antral origin elicited gastric acid secretion when intravenously injected into cats, confirmed the existence of gastrin.

1.9.1 Chemical structure

Establishment of the amino acid sequence of gastrin (Gregory, Hardy, Jones, Kenner and Sheppard, 1964) followed the isolation and purification of gastrin from porcine gastric mucosa (Gregory and Tracy, 1964). A synthetic replicate of the peptide isolated from the gastric antrum (Anderson, Barton, Gregory, Hardy, Kenner, MacLeod, Preston, Sheppard and Morley, 1964) was reported to have similar biological properties to the natural hormone (Gregory and Tracy, 1964; Morley, Tracy and Gregory, 1965). Isolation of gastrins from many animal species has been achieved (review by Walsh, 1994); it has been established unequivocally that mammalian gastrins share in common the carboxy-terminal tetrapeptide amide sequence, tryptophan-methionine-aspartate-phenylalanine amide. This has been shown to be the active site of the peptide and is required for the full expression of the biological activity of gastrin (Morley, Tracy and Gregory, 1965). For this reason, synthetic pentagastrin (containing the terminal 5 amino acid residues of gastrin) is often used in clinical tests, since it is easier to synthesise than the full molecule.

The application of radioimmunoassay (RIA) has allowed measurements of circulating gastrin levels to be recorded. This was first achieved for gastrin in 1968 by McGuigan and Trudeau who reported resting levels in fasted human subjects (with no known gastrointestinal disorder) of between 245 and 668pg ml^{-1} of plasma. However, over the following two years, documented resting levels of gastrin have been much lower; one study reported levels of between 5 and 290pg ml^{-1} (Hansky and Cain, 1969), and another suggested levels in control human subjects were no higher

than 300pg ml^{-1} (Yalow and Berson, 1970). Hansky and Cain proposed that the disparity between the initial report and their study could be accounted for by differences in vagal tone. Later, in the early 70s, reported levels fell further such that mean values of 71pg ml^{-1} (McGuigan and Trudeau, 1973) and 44pg ml^{-1} (Straus, Gerson and Yalow, 1974) were announced. However, with advances in RIA enabling improved sensitivity, resting levels of gastrin greater than 20pg ml^{-1} are only considered to occur in approximately one quarter of subjects with no apparent gastrointestinal disease (reviewed by Straus, 1978).

Several forms of gastrin exist. Following the isolation of two heptadecapeptide gastrins (G17) (both composed of 17 amino acid residues but in which the tyrosine at position 6 was sulphated or non-sulphated) from porcine antral mucosa (Gregory and Tracy, 1964), further studies have established the existence of different forms of gastrin in a variety of species. The presence of gastrin heptadecapeptides and hexadecapeptides in a number of mammals such as the dog (Bonato, Eng, Hulmes, Miedel, Pan and Yalow, 1986), cat (Agarwal, Kenner and Sheppard, 1969), sheep and cow (Agarwal, Beacham, Bentley, Gregory, Kenner, Sheppard and Tracy, 1968), in addition to human (Bentley, Kenner and Sheppard, 1966) and rat (Reeve, Dimaline, Shively, Hawke, Chew and Walsh, 1981) has been documented (Fig. 1-9).

man	Glu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe
pig	Glu-Gly-Pro-Trp-**Met**-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe
cow	Glu-Gly-Pro-Trp-**Val**-Glu-Glu-Glu-Glu-**Ala**-Ala-Tyr-Gly-Trp-Met-Asp-Phe
rat	Glu-**Arg**-Pro-**Pro**-**Met**-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe
dog	Glu-Gly-Pro-Trp-**Met**-Glu-Glu-**Ala**-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe

Fig. 1-9: Gastrin heptadecapeptide (G17) structures in man, pig, cow, rat and dog. Amino acid residues are indicated by conventional three letter codes. Amino acid residues in bold denote structural differences compared with human G17. Phe (phenylalanine) is present as amide. (Taken from Andersen, 1985).

Sequences were subsequently reported for pig and human which showed the presence of another species of gastrin, "big gastrin", a dimer of G17 containing 34 amino acid residues (G34) (Gregory and Tracy, 1975). However, it has since been shown that in the mammalian species studied (humans, cows, sheep, pigs, dogs, cats, rats, rabbits, guinea pigs, mice), only about 5% of gastrin was G34 (Andersen, 1985). This

confirms the work of Berson and Yalow (1971), who determined that, in man, more than 90% of antral gastrin is G17. In the guinea pig, a form of big gastrin has been reported which differs from pig G34 in 6 of the 17 NH_2-terminal amino acids, as well as in the deletion of a glutamic acid in the carboxy-terminus; it is therefore referred to as G33 (Bonato, Eng, Pan, Miedel, Hulmes and Yalow, 1986). Furthermore, it was discovered that human duodenum and antrum contain very similar amounts of gastrin activity. By contrast, in other species including the dog and the pig, approximately 98% of gastrin is found in the antrum (Nilsson, Yalow and Berson, 1973). Support for this comes from Feurle, Menzel and Klempa (1983), who determined that, in the dog, less than 4% of circulating gastrin was contributed to by the duodenum. The potency for the stimulation of gastric acid secretion in response to either G34 or G17 in man is very similar (Eysselein, Maxwell, Reedy, Wünsch and Walsh, 1984); however, the half-time clearance rates for G17 and G34 differ considerably. The clearance rate of G17 from the human circulation is approximately six minutes, whereas that for G34 is about six times longer (Walsh, Debas and Grossman, 1974; Walsh, Isenberg, Ansfield and Maxwell, 1976).

1.9.2 Gastrin release

In the stomach, gastrin is released from antral G cells, which are found distributed with enterochromaffin, mucus-containing and somatostatin-containing D cells. G cells are open type endocrine cells which are thought to sample luminal contents at their apical membrane (in chapter by Lloyd and Walsh, 1994).

There are three phases of gastric acid secretion, in which gastrin plays a major role. These are the cephalic, gastric and intestinal phases.

1.9.2(i) *Cephalic phase*

This refers to the period prior to food reaching the stomach, whereby acid secretion is enhanced simply by the thought, taste, smell, sight and swallowing of food. This response is mediated entirely by the vagus nerves (Pe Thein and Schofield, 1959), by way of direct cholinergic stimulation of the parietal cell, and by non-cholinergic (GRP-induced) release of gastrin from the G cells (as summarised by Lloyd and Walsh, 1994). During the cephalic phase, the rate of acid secretion may be as high as 40% of the maximal rate. However, in the absence of the buffering effect of food with

the resultant inhibition of activation of gastrin release elicited by low pH in the antrum, the actual amount of secreted acid is small (chapter by Kutchai, 1998).

1.9.2(ii) *Gastric phase*

Gastric acid secretion during this phase occurs in response to both distension of the stomach, and through the actions of partially digested proteins (peptides and amino acids) on the antral mucosa to cause gastrin release (Taylor, Byrne, Christie, Ament and Walsh, 1982; McArthur, Isenberg, Hogan and Dreier, 1983). The most potent effectors are the aromatic amino acids, tryptophan and phenylalanine (Taylor *et al*, 1982). Distension of either the body or antrum of the stomach results in the stimulation of mechanoreceptors in the gastric wall, and subsequent elicitation of a vago-vagal reflex. This is characterised by excitation of afferent vagal fibres from the stomach which pass to the nucleus of the tract solitarius in the medulla oblongata. A neural projection from here to the dorsal vagal nucleus, and subsequent excitation of pre-ganglionic fibres, results in the release of acetylcholine from post-ganglionic vagal fibres in the gastric mucosa. Acetylcholine acts on the parietal cell, both directly and indirectly through stimulation of gastrin release from G cells (see chapter by Kutchai, 1998). Although GRP plays a part in the cephalic phase of acid secretion, it does not appear to play any significant role in distension-induced gastric acid secretion (Kovacs, Lloyd, Miller, Coy and Walsh, 1989). More recently, a study has documented the involvement of vasoactive intestinal peptide (VIP) in gastrin secretion induced by gastric distension in the rat (Schubert and Makhlouf, 1993). Low distension resulted in a decrease in gastrin release and increased somatostatin release, whereas high distension enhanced gastrin release and reduced somatostatin release. Moreover, administration of atropine had no effect on the response to low distension, but caused reversal of the response to high distension, that is, gastrin release was reduced and somatostatin release increased. VIPergic neurones are activated in response to both low and high distension. However, in the presence of a selective VIP antagonist, both the gastrin and the somatostatin response to low distension were abolished. The authors concluded that VIP neurons were activated by low distension; VIP stimulates somatostatin release which subsequently inhibits the release of gastrin. As distension increased, progressive recruitment of cholinergic neurons resulted, and consequently, gastrin release increased.

The interactions between gastrin and vagal stimulation are of considerable significance since gastric acid secretion in response to gastrin is markedly potentiated by the basal secretion of acetylcholine in an innervated (Pavlov) pouch, when compared to that in a denervated (Heidenhain) pouch (Andersson and Grossman, 1965) (Fig. 1-10).

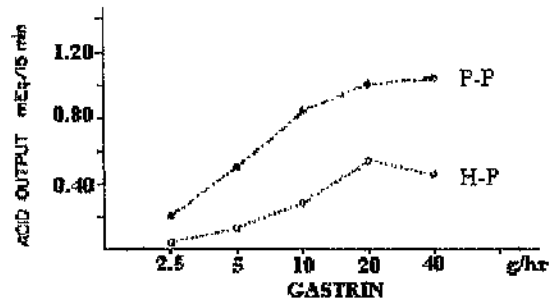


Fig. 1-10: Synergistic effect of vagal innervation on gastrin, as demonstrated by comparison of the responses to exogenous gastrin (I.V.) in the Pavlov pouch (innervated) (P-P) and the Heidenhain pouch (denervated) (H-P) in the dog. (Taken from Andersson and Grossman, 1965).

Gastrin accounts for most of the acid secreted in response to luminal peptides and amino acids, as determined in a study measuring circulating concentrations of gastrin in response to graded increases in intragastric concentrations of peptones (Maxwell, Eysselein, Kleibeuker, Reedy and Walsh, 1984).

Other chemical components that cause release of gastrin and enhance gastric acid secretion in man include decaffeinated coffee (Feldman, Isenberg and Grossman, 1981) (Wright, Gibson and Hirschowitz (1977) have demonstrated that caffeine itself does not evoke gastrin release) and fermented alcoholic beverages (not distilled) (Lenz, Ferrari-Taylor and Isenberg, 1983; Singer, Teyssen and Eysselein, 1991; Teyssen, Lenzing, González-Calero, Korn, Riepl and Singer, 1997). The stimulation of gastrin release by fermented alcoholic beverages has been additionally shown in rats (Teyssen, González-Calero, Korn and Singer, 1997).

1.9.3(iii) *Intestinal phase*

This is elicited by the presence of chyme in the duodenum, which brings about neural and endocrine responses which in the first instance stimulate but, later, inhibit gastric acid output (chapter by Kutchai, 1998).

Stimulation of secretion occurs through distension of the duodenum, and by the presence of peptides and amino acids. Distension-induced secretion occurs by way of a vago-vagal reflex in much the same way as mechanical stimulation of the stomach, whereas chemical stimulation occurs by way of duodenal and jejunal G cell activation. A study by Ayalon, Devitt, Guzman, Suddith, Rayford and Thompson (1982), whereby 10% liver extract was instilled into the duodenum and proximal jejunum of dogs with innervated antral pouches, reported significantly increased levels of gastrin in both gastric venous blood and systemic blood. This lends support to the idea that chemical stimulation causes gastrin release during the intestinal phase.

Kosaka and Lim (1930a) established that lipid in the small intestine of the dog caused antagonism of gastric secretion by the release of inhibitory substances. In addition to luminal lipid, acid, hyperosmolar solutions and elevated intraluminal pressure within the intestinal lumen are implicated in the inhibition of gastric acid secretion. The substances responsible for inhibition of acid secretion have still not been fully established, and are thus referred to as enterogastrones (as derived by Kosaka and Lim, 1930b). Peptide YY is likely to be one mediator since plasma concentrations of peptide YY in the circulation increase significantly in response to a meal (Taylor, 1985). Moreover, peptide YY is released from the small intestine in response to lipid perfusion of the distal small intestine in dogs (Aponte, Fink, Meyer, Tatemoto and Taylor, 1985), and administration of exogenous peptide YY, in doses that mimic plasma levels after a meal, results in inhibition of gastric acid secretion (Pappas, Debas and Taylor, 1986).

The presence of acid in the duodenum is thought to inhibit gastric acid secretion through release of secretin (as summarised by Lloyd and Debas, 1994), although inhibition of gastric acid secretion has not always been reproduced by infusion of exogenous secretin in physiological doses (Kleibueker, Eysselein, Maxwell and Walsh, 1984).

To further complicate matters, a study by Orloff, Bunnett, Wong, Walsh and Debas (1991), investigating the neural and hormonal mechanisms involved in the enterogastric reflex, proposed that somatostatin and CCK were the principal enterogastrones. CCK is a gastrointestinal hormone with an amidated carboxy-terminal pentapeptide identical to that of gastrin. CCK, thus, possesses some gastrin-

like attributes, although with considerably less potency, and may thus act as a competitive inhibitor of gastrin (for full review consult Liddle, 1994).

Thus, one mediator of the enterogastrone reflex appears to be peptide YY; in addition, secretin, CCK and/or somatostatin may also play a role. However, the need for further work in this field is apparent.

1.9.4 Mechanism of gastrin-induced gastric acid secretion

Two separate mechanisms for the activation of gastric acid secretion by gastrin have been proposed by Code (1965) and by Grossman (1967). In the former, gastrin was hypothesised to act by stimulating the release of histamine from enterochromaffin-like (ECL) cells, which subsequently acts on the parietal cells. In the latter, the direct activation of the parietal cells by gastrin to stimulate gastric acid secretion, was proposed.

1.9.4(i) *Histamine as the final common mediator*

It was suggested that gastrin may act to induce gastric acid secretion by stimulating the release of histamine from ECL cells of the stomach. Subsequent activation of the parietal cells by histamine follows (Chuang, Chen and Soll, 1991), mediated by increased levels of intracellular adenosine 3'-5'-cyclic monophosphate (cAMP) (Soll and Wollin, 1979). Endorsement of the view that histamine may be a final common mediator has come from a number of studies: histamine is in wide abundance in gastric tissue (Lönroth, Hakanson, Lundell and Sundler, 1990); administration of histamine receptor antagonists in rats attenuated the acid stimulating effect of gastrin, as well as that of histamine (Lloyd *et al*, 1992); gastrin has been implicated in the regulation of the expression and activity of histidine carboxylase, the enzyme responsible for the production of histamine (Dimaline and Sandvik, 1991). These studies thus indicate that gastrin-induced gastric acid secretion may be mediated through the action of histamine on the parietal cell.

1.9.4(ii) *Direct action of gastrin on the parietal cell*

The alternative view is a direct action of gastrin on the parietal cells, with no involvement of histamine. Synergism between the activities of gastrin and histamine in the enhancement of gastric acid secretion in the dog has been demonstrated

(Andersson and Grossman, 1965), which is consistent with the concept that the action of gastrin on the parietal cells occurs by mediation of intracellular pathways separate from those mediating the effect of histamine. The apparent inhibition of the response to gastrin in the presence of histamine receptor antagonists (Lloyd *et al*, 1992) presumably reflects withdrawal of histamine enhancement of gastrin action (Soll, 1978a; Soll, 1982).

In an investigation aimed at resolving whether gastrin acts through histamine release, Blair (1966) reported that, in anaesthetised cats, neither the histamine output in gastric washings nor the histamine concentration in gastric venous blood, following gastrin-stimulated secretion of gastric acid, increased to the level which occurred when histamine caused the same level of gastric acid secretion. Subsequent studies using radioligand binding (Soll, Amirian, Thomas, Reedy and Elashoff, 1984), and mammalian cell culture techniques (Soll, 1978a; 1978b; 1980), in addition to further reports of the potentiation existing between gastrin and histamine (Soll, 1978a; Soll and Grossman, 1981) have established a direct action of gastrin on the parietal cell, and it is now generally accepted that histamine release from ECL cells and direct parietal cell stimulation are both separate physiological actions of gastrin. The mechanism by which gastrin induces its direct action is described more fully below.

Gastrin enhances acid secretion by binding to receptors, denoted as CCK-B, on the parietal cell, initiating a series of cascade events (Fig. 1-11). (Acetylcholine, through the activation of muscarinic receptors on the parietal cell membrane, initiates a similar series of events.) Occupation of the receptor on canine, porcine or guinea pig parietal cells activates a membrane-bound enzyme, phospholipase C (PLC), which catalyses the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into two components, inositol trisphosphate (IP_3) and diacylglycerol (DAG). Ca^{2+} release from intracellular stores, and influx of extracellular Ca^{2+}, by the action of IP_3, results in increase levels of free intracellular calcium. DAG, accompanying these increased Ca^{2+} levels, stimulates the translocation of protein kinase C (PKC) from the cytoplasm to the cell membrane, where it is activated (Chiba, Fisher, Park, Seguin, Agranoff and Yamada, 1988; Tsunoda, Takeda, Otaki, Asaka, Nakagaki and Sasaki, 1988; Tsunoda, Takeda, Asaka, Nakagaki and Sasaki, 1988; Tsunoda, Yodozawa and Tashiro, 1989; Cabero, Rehfeld and Mårdh, 1989; DelValle, Tsunoda, Williams and Yamada, 1992). Phosphorylation of proteins (which are still to be elucidated, but may involve the

H^+/K^+ ATPase) by PKC occurs, and the result is an increase in gastric acid production (as reviewed by Yamada, Chiba, DelValle and Campbell, 1993).

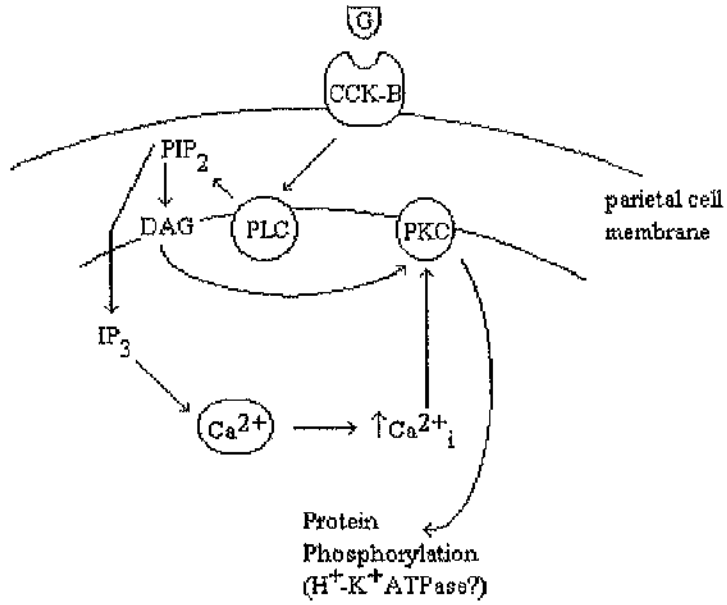


Fig. 1-11: Proposed mechanism of gastrin-induced gastric acid secretion. G, gastrin; CCK-B, gastrin receptor; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-biphosphate; DAG, diacylglycerol; IP_3, inositol trisphosphate; PKC, phosphokinase C.

1.10 Rationale of the present study

The tetrapeptide of gastrin containing the terminal four amino acids (called tetragastrin) was the hormone chosen for transport across the small intestine. In these experiments, gastric acid levels were measured in the fasted anaesthetised rat, *in vivo*, as a bioassay for the levels of tetragastrin in the bloodstream.

The first step in the present study was therefore the synthesis of the tetragastrin-cholic acid conjugate (G-CA), whereby tetragastrin was coupled at the C-24 position of cholic acid. Subsequently, the purity of this compound needed to be determined, and its biological activity confirmed.

As a prelude to the main experiments investigating the intestinal absorption of G-CA, it was necessary to carry out control experiments to test whether there was ileal absorption of tetragastrin, *per se*. This could then be compared with the results from

the experiments with G-CA to determine the extent to which the conjugation of tetragastrin with cholic acid enhanced absorption. Additionally, it would be necessary to investigate the site specificity of the response by testing the transmucosal absorption of G-CA across the jejunal wall. This would provide an indication of whether the ileal bile salt transporters were being utilised.

METHODS

2.1 Physiological experiments

Experiments were carried out on 86 anaesthetised male Wistar rats with body weights of between 250 and 350g. The rats were starved overnight but had access to a 5% glucose drink. The duration of the experiments during which gastric secretions were collected ranged between 300 and 675 minutes.

2.1.1 Surgical procedures

2.1.1(i) *Anaesthesia*

Anaesthesia was induced with an I.P. injection of sodium pentobarbitone (Sagatal, Rhône Merieux, Dublin; 80mg kg^{-1}). The criterion for anaesthesia was abolition of the hind limb flexor withdrawal reflex. Subsequently, routine administration of 0.1ml sodium pentobarbitone (I.M.) was carried out every hour in order to maintain the level of anaesthesia. In the event of return of the flexor withdrawal response, anaesthesia was restored promptly with 4% halothane (May and Baker Ltd., Dagenham) in oxygen (1 litre min^{-1}), and an additional injection of 0.1ml sodium pentobarbitone (I.M.) was made. Once the venous cannula had been inserted, return of the withdrawal response was abolished by intravenous injection of 0.1ml sodium pentobarbitone (allowing for dead space in the cannula and tap).

2.1.1(ii) *Tracheal and blood vessel cannulations*

Tracheostomy was carried out on each rat to allow for artificial ventilation if required (using the Harvard rodent ventilator, 100% oxygen, stroke of 1.0ml $100g^{-1}$, rate of 60ml min^{-1}). The carotid artery was cannulated with a cannula (Portex, 1.02mm outer diameter, pink luer) containing 2% heparin (Multiparin, CP Pharmaceuticals Ltd., Wrexham) in isotonic saline. This was connected to a transducer and amplifier which led to a Lectromed 4 chart recorder, allowing blood pressure to be monitored. The external jugular vein was then cannulated with a cannula (Portex, 0.75mm outer diameter, blue luer) containing 2% heparin in isotonic saline to allow for the slow bolus infusion of drugs, which was done over a period of 1.0min.

2.1.1(iii) *Gastric cannulation*

A midline incision of the abdominal wall was made from the xiphoid cartilage to 5cm caudally. The oesophagus was tied off at the oesophago-gastric junction to prevent reflux of the gastric contents. This also effected a bilateral vagotomy since the vagus nerves passed over the oesophagus at this point. Care was taken to prevent occlusion of the left gastric vein running alongside the oesophagus. A small incision was made in the duodenum near the pyloro-duodenal junction, and a perforated polythene stomach tube (outer diameter of 3.0mm) was inserted into the stomach. This was tied firmly in place, and a mosquito clip was used to close the abdominal wall around the stomach tube. A thermometer was inserted into the rectum, and body temperature was maintained between 35 and 37°C using two external tungsten lamps.

The stomach contents were washed out by infusion and withdrawal, via the perforated stomach tube, of 1.0ml glycine/mannitol buffer (see Section 2.1.2 for composition). When the washouts were clear, a perforated cannula (Portex, 1.65mm outer diameter, red luer) was pushed into place within the stomach tube, and a 2-way tap was inserted into the end of the cannula. The stomach tube was supported in the upright position with a clamp. Figure 2-1 illustrates the experimental set-up.

2.1.1(iv) *Ileal cannulation*

When drugs were to be administered into the ileum, just prior to ileal infusion, a small incision was made close to the ileo-caecal junction, and a short length of cannula (Portex, 1.65mm outer diameter, red luer) inserted in the orad direction, such that drugs would be infused towards the proximal small intestine. This cannula was tied firmly in place.

2.1.1(v) *Jejunal cannulation*

In the case of jejunal drug administration, a small incision was made close to the ligament of Treitz, and a short length of cannula (Portex, 1.65mm outer diameter, red luer) was inserted in the aboral direction such that drugs would be infused distally. A further ligature was tied in place, 15cm distal to the jejunal cannula, ensuring that absorption from the jejunum and not the ileum was being investigated.

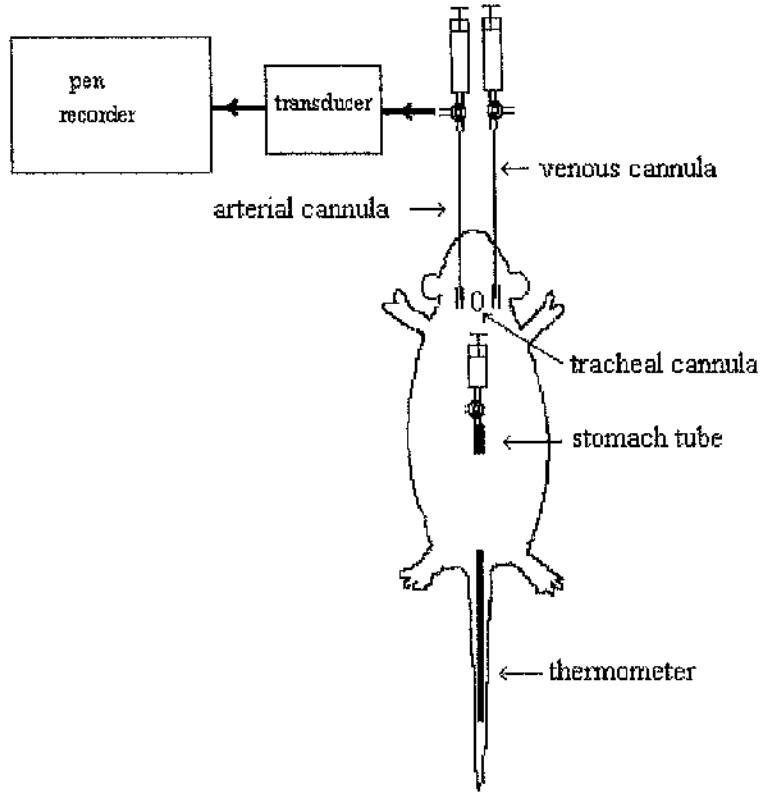


Fig.2-1: Schematic representation of the experimental set-up for the collection of gastric acid secretions in the anaesthetised rat.

2.1.2 Composition of glycine/mannitol buffer

This was made up as follows:

1 part glycine (0.3M) (AnalaR)

4 parts D-mannitol (0.3M) (Sigma Chemical Co., M-9647)

The pH of the buffer was adjusted to 6.5 by addition of NaOH (1.0M).

2.1.3 Substances administered *in vivo*

The following substances were used in the physiological experiments

- Trp-Met-Asp-Phe amide (tetragastrin), 99% peptide content (Sigma Chemical Co. T-6515).
- Cholate-Trp-Met-Asp-Phe amide conjugate (G-CA) (synthesised *de novo* using Pioneer Peptide Synthesis System, PerSeptive Biosystems (see Section 2.3)).
- Glycocholic acid (N-[3α, 7α, 12α-trihydroxy-24-oxocholan-24-yl] glycine) (Sigma Chemical Co. G-7132).

Both tetragastrin and G-CA were stored in anhydrous form at less than 0°C until required. The doses are expressed in terms of weight of peptide per body weight, *viz.* $\mu g\ kg^{-1}$.

2.1.4 Measurements

2.1.4(i) *Measurement of pH of gastric secretions*

Gastric secretions were collected over 15 minute periods. Initially, 1.0ml glycine/mannitol buffer (pH adjusted to 6.5) was injected into the stomach via the stomach tube; this was withdrawn after 15 min, and replaced with 1.0ml fresh buffer at 37°C. The volume withdrawn was noted, and the pH of the sample measured immediately. The pKa of the buffer was 3.5, and so any H^+ secretion was readily detectable as a fall in the pH of the sample from pH 6.5. The sample was titrated directly to pH 6.5 with 100mM or 10mM NaOH, as appropriate, using a precision 100µl syringe and continuous stirring. The volume of NaOH required to do this was noted, and conversion of this volume into mequiv. HCl was then carried out. As a precaution, the NaOH solutions were stoppered at all times to prevent the absorption of CO_2, and the concentration was checked daily by titration against a precise HCl standard.

2.1.4(ii) *Measurement of blood pressure*

The blood pressure of each animal under investigation was routinely recorded throughout the experiments, thereby allowing us to constantly monitor its condition. This was of particular importance in the experiments in which an absence of biological activity of administered compounds occurred.

Values for the systolic and diastolic blood pressure (SBP and DBP, respectively) of each individual animal prior to, subsequent to, and 60, 90 or 180 minutes (depending on the time period before the next procedure) after the administration of test compounds, were obtained, and mean arterial blood pressure (MABP) was calculated as: MABP = 1/3 (SBP - DBP) + DBP.

For ease of comparison, mean arterial blood pressure for every procedure in each series of experiments was expressed as mean ± S.D. (standard deviation).

2.1.4(iii) *pH measurements of administered agents*

In order to provide a more detailed evaluation of the processes involved in the transport or lack of transport of intestinally-administered agents, the pH values of all solutions were measured prior to administration. These values, together with known pKa values, enabled us to determine, with some confidence, the state of ionisation of the administered compounds, and thus describe their ability or inability to move through the partition of the lipid membranes (see Appendix).

2.1.5 Analysis of results

The responses to the experimental procedures were expressed in terms of the absolute increase in total acidity above baseline levels, that is (response - baseline). Gastric acid levels very often fell during the initial collection periods; however, a steady baseline value was attained, usually over a period of between 45 and 60 minutes (3 or 4 collection periods). Then the experimental procedures commenced. Hence, the baseline was calculated as the average gastric acid secretion over these 3 or 4 collection periods prior to drug administration.

Also calculated were the cumulative increases in gastric acid secretion above baseline over the hour including and following drug administration. A fixed time period was decided upon rather than attempt to measure the total acid secretion of the entire response (which often remained elevated for longer than one hour) since, in many cases, the gastric acid levels did not return to the same baseline value preceding experimental procedures, but rather remained at slightly elevated levels.

It was decided against calculation of the factor by which gastric acid secretion increased above baseline in response to experimental procedures. The same absolute change in total acidity would present a different factor increase above baseline if the baseline varied in the two cases. Since many comparisons were made in response to the same procedure repeated in the same animal, i.e. paired procedures, to introduce the possibility of differences due to the method of expression of the results was deemed unacceptable.

The paired t-test against zero was employed to compare between two procedures in the same rat, and the two sample t-test for the comparison of identical procedures in different populations of rats. Occasionally the data which could otherwise be judged to conform to a normal distribution were skewed by one or two unusually large

values. In these cases, two procedures were adopted. First, the t-test was undertaken after omitting the unusually large values. This was deemed justifiable since the t-test is the most rigorous method of comparing two populations which conform to normal distribution, i.e. have a mean and standard deviation. Second, the entire data were compared with the Wilcoxon statistical test, a non-parametric test. This test is based on the relative rankings of the individual data and compares the medians of the population. These tests were undertaken using Minitab for Windows version 10 (Ryan and Joiner, 1994). Statistical significance was taken as $P<0.05$.

2.2 Histological studies

An additional study was undertaken to determine whether infusion of G-CA had caused any adverse structural changes to the intestinal mucosa. Following completion of the experimental procedures, ileal tissue from two rats was excised and prepared for light microscopic evaluation. Similarly, slices of ileal tissue from two control animals, in which intra-ileal infusion of G-CA was not undertaken, were removed for comparison.

2.2.1 Fixation and staining procedures

Sections of ileum, 2.5cm long, were fixed overnight in 10% neutral phosphate-buffered formalin at pH 7.4. After fixation, the formalin was washed from the sample, the tissue divided into smaller pieces and dehydrated by passing it through a graded series of alcohol solutions up to 100 percent, and then through chloroform. Next, the tissue was passed through xylene, a nonaqueous liquid miscible with paraffin wax, and embedded in paraffin wax (Paraplast). The tissue blocks were sectioned on a Leitz rotary microtome to produce 5μm thick sections, which were mounted on albumin coated slides.

For staining, the paraffin wax was dissolved with xylene, and the sections were rehydrated by passing them through a graded series of alcohol solutions back to water. The sections were stained with Gill's Haematoxylin and 1% aqueous Eosin (H&E) and dehydrated as described above. Finally, the sections were mounted in DPX (Distrene 80 Dibutyl Phthalate Xylene).

2.3 Chemical synthesis of tetragastrin-cholic acid conjugate

The successful manufacture of a tetragastrin-cholic acid conjugate is central to the outcome of this project. The following account describes the steps undertaken in order to achieve this.

2.3.1 Main chemical substances used in synthesis of conjugate

2.3.1(i) *Reagents*

- Glycinamide hydrochloride (Sigma Chemical Co. G-7378)
- 3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid (Cholic acid) (Sigma Chemical Co. C-1129)
- N, N'-Dicyclohexylcarbodiimide (DCC) (Sigma Chemical Co. D-1328)
- N-Hydroxy-2, 5-pyrrolidinedione (N-hydroxysuccinimide) (Sigma Chemical Co. H-7377)
- Trp-Met-Asp-Phe amide (Tetragastrin) (Sigma Chemical Co. T-6515)
- Amino acid derivatives: tryptophan, methionine, aspartate, phenylalanine (PerSeptive Biosystems Inc.)

2.3.1(ii) *Solvents*

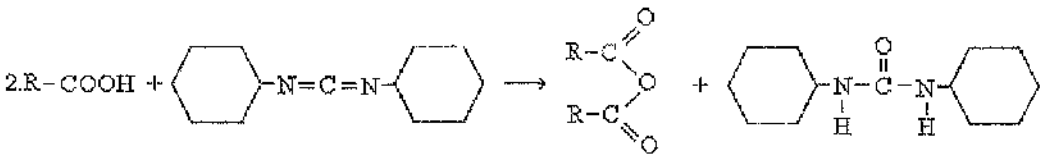
- Butanol (Fisher Scientific UK Ltd.)
- Glacial acetic acid (Fisher Scientific UK Ltd.)
- Dichloromethane (Aldrich Chemical Co. Ltd.)
- Dimethyl sulphoxide (DMSO) (Sigma Chemical Co. D-5879)
- Acetonitrile (methyl cyanide) (Sigma Chemical Co. A-6914)
- N, N-Dimethyl formamide (Sigma Chemical Co. D-8654)
- N-methyl morpholine (Rathburn Chemicals Ltd.)

2.3.1(iii) *Staining reagents / miscellaneous*

- Iodine crystals (AnalaR)
- 1,2,3-Indantrione (Ninhydrin) (AnalaR)
- ρ-Dimethylaminobenzaldehyde (AnalaR)
- N-[3α, 7α, 12α-Trihydroxy-24-oxocholan-24-yl] glycine (Glycocholic acid) (Sigma Chemical Co. G-7132)

2.3.2 Background theory to conjugation reaction

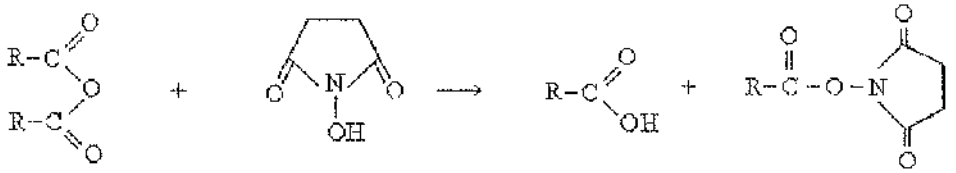
Combination of cholic acid (represented by RCOOH) and N, N'-Dicyclohexylcarbodiimide (DCC) results in the formation of a cholic acid anhydride, and the insoluble by-product, N, N'-Dicyclohexylurea (DCU), formed by the removal of water from cholic acid by DCC (equation 2-1). The resultant cholic acid anhydride is a fairly good acylating reagent (acylation is the substitution of a hydrogen atom by an acyl[1] group). Removal of DCU is carried out by filtration.



cholic acid | DCC | cholic acid anhydride | DCU

(Equation 2-1)

In the presence of N-hydroxysuccinimide, each molecule of cholic acid anhydride is converted to one molecule of N-hydroxysuccinimide ester and one molecule of cholic acid (equation 2-2). The N-hydroxysuccinimide esters are far better acylating agents than the acid anhydride.

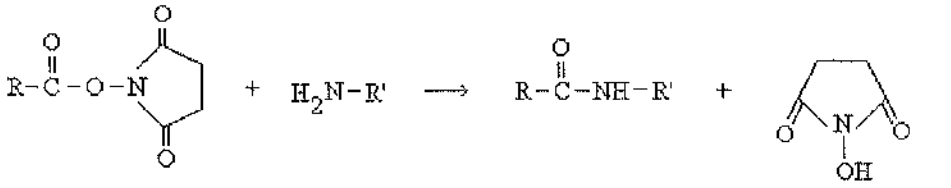


cholic acid anhydride | N-hydroxysuccinimide | cholic acid | N-hydroxysuccinimide active ester

(Equation 2-2)

[1] *The general term for an acyl group is R-CO- .*

The N-hydroxysuccinimide ester is highly reactive, and when in combination with the chosen peptide (represented by H_2N-R') , should readily yield the cholic acid-peptide conjugate (equation 2-3). Furthermore, the easy removal of the water soluble by-product, N-hydroxysuccinimide, makes the process all the more suitable.

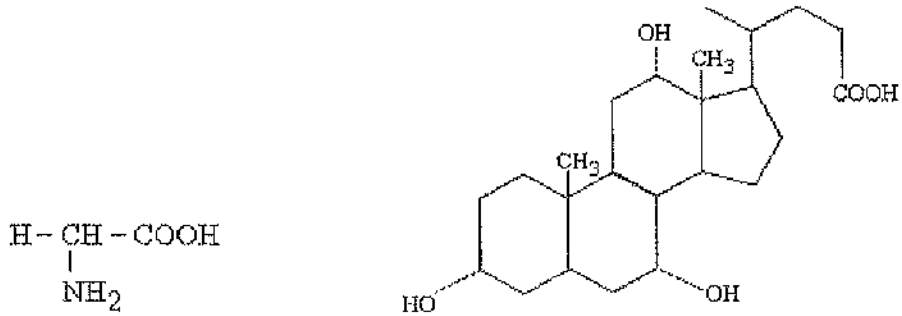


N-hydroxysuccinimide active ester | peptide | peptide-cholic acid conjugate | N-hydroxysuccinimide

(Equation 2-3)

2.3.3 Glycine-cholic acid conjugation

It was decided to establish first the success of the conjugation procedure by carrying out the simplest reaction, that is, the conjugation between an amino acid and a bile acid. The amino acid chosen was glycine, and the bile acid was cholic acid, thereby enabling a comparison to be made between the attempted conjugation of these two compounds with a glycocholic acid standard solution. The chemical structures for both glycine and cholic acid are illustrated in Fig 2-2.



(a) (b)

Fig. 2-2: Chemical structures of (a) glycine and (b) cholic acid.

2.3.3(i) *Thin layer chromatography (TLC)*

The technique employed initially in the determination of the success of the conjugation reaction was thin layer chromatography (TLC). This is a separation method, and can be used simply to check the purity of a substance, to attempt to separate and identify the components in a mixture, or to obtain a quantitative analysis of one or more of the compounds present.

In practice, the sample to be separated is applied on the layer, 1-2 cm from one end of the plate (known as the starting point or origin), and separation is achieved by passing a solvent (the mobile phase) through the layer. After the front of the mobile phase has reached to within a short distance from the top of the layer, the plate is removed and allowed to dry, before carrying out detection procedures.

The Rf value provides a useful way of expressing the position of the separated components of a sample on a developed chromatogram. It is calculated as the ratio:

$$Rf = \frac{\text{distance from origin to centre of separated zone}}{\text{distance from origin to solvent front}}$$

Accordingly Rf values fall between 0.0 and 1.0.

It was necessary to establish first the most suitable sorbent (layer) and mobile phase to use. Silica gel plates (Silica gel 60, Merck) were selected since they are suitable for TLC of amino acids and bile acids. Butanol-glacial acetic acid-water (120 + 10 + 10) was chosen as the mobile phase, since it was discovered that TLC of cholic acid (10mg ml^{-1} methanol), glycine (10mg ml^{-1} methanol/water (50/50)) and glycocholic acid (10mg ml^{-1} methanol) resulted in a satisfactory separation between spots of these compounds (Fig. 2-3) (Rf values of cholic acid, glycocholic acid and glycine were 0.833, 0.522 and 0.044, respectively). This was deemed necessary since, when comparing TLC of these standards with TLC of the conjugation sample, less ambiguity as to whether conjugation had or had not been achieved would arise. TLC was run for a period of 2 hours, the plate allowed to dry, and unless otherwise stated, stained with iodine crystals, a universal stain.

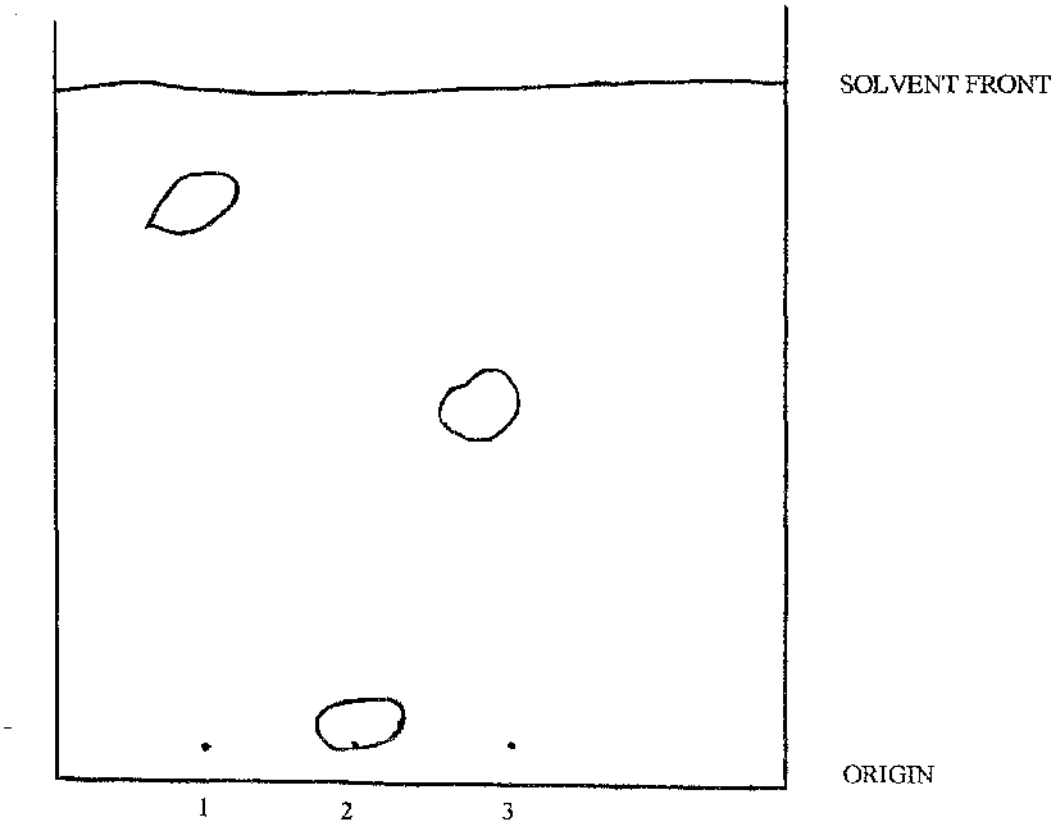


Fig. 2-3: Thin layer chromatography of: (1) cholic acid, (2) glycine and (3) glycocholic acid (all 20µl spots; 10mg ml^{-1}) on Silica Gel G layer, developed with butanol-glacial acetic acid-water (120+10+10) for 2 hours, and detected with iodine crystals. Exact tracing of developed TLC plate.

2.3.3(ii) *Selection of solvent*

The procedure for conjugation of an amino acid or a peptide with cholic acid was carried out at room temperature (20^{0}C), and required that the individual reactants be dissolved in a non-aqueous solvent, since water vapour could react with the active intermediary (N-hydroxysuccinimide active ester; equation 2-3).

Our first choice of non-aqueous solvent was dichloromethane; however, it was discovered that cholic acid, glycine and N-hydroxysuccinimide were all insoluble in it. Dimethyl sulphoxide (DMSO) was found to dissolve all components of the conjugation reaction, except glycine. An attempt to increase the solubility of glycine in DMSO by the addition of a highly polar solvent, N-methyl-morpholine, to produce a final solution of glycine in 1:1 N-methyl-morpholine:DMSO was not successful.

Furthermore, increasing the N-methyl-morpholine ratio 10-fold (that is, glycine in 10:1 N-methyl-morpholine:DMSO) did not result in an increase in glycine solubility.

Finally, dimethyl formamide was selected as a possible non-aqueous solvent. However, similar problems were found, as with DMSO, regarding the insolubility of glycine, despite the relative solubility of cholic acid, DCC and N-hydroxysuccinimide. As described previously, addition of N-methyl-morpholine to the glycine/dimethyl formamide solution, on a 1:1 basis, did not increase solubility; neither did increasing N-methyl-morpholine concentration on a 10:1 basis.

The insolubility of glycine was considered to be as a result of its very polar nature. It was anticipated that the solubility of the test peptide to be conjugated at a later stage would be less problematic, since it would have a less polar nature as a consequence of the presence of aromatic groups, thereby reducing the zwitterion effect.

However, despite the insolubility of glycine in dimethyl formamide, it was decided to attempt the conjugation of glycine with cholic acid. 200mM solutions/suspensions of each individual reactant in dimethyl formamide were made up.

2.3.3(iii) *The conjugation procedure*

The conjugation between cholic acid and glycine was attempted by performing the following procedure. Equal volumes of the cholic acid solution and the DCC solution were mixed together. To this was added an equal volume of the N-hydroxysuccinimide solution, followed immediately by the addition of the same volume of the glycine suspension. Immediate mixing was required in order to minimise water vapour reacting with the active intermediary yielded by the reaction between the cholic acid, DCC and N-hydroxysuccinimide solutions.

Since glycine was dispersed rather than dissolved in dimethyl formamide, on addition of it to the reaction vessel (containing cholic acid, DCC and N-hydroxysuccinimide solutions), some glycine crystals were left remaining in the original vessel (that is, the vessel in which the glycine dispersion was made up). In an attempt to ensure that all the glycine was available for the reaction, the conjugation mixture (contained in reaction vessel) was poured back and forth between the original and the reaction vessel. Finally, the conjugation mixture was divided into two; half was placed in the reaction vessel, and half was placed in the original vessel. These two vessels, labelled

'con 1' and 'con 2', respectively, were stoppered and vigorously shaken for approximately 3 hours, before further analysis was carried out.

2.3.3(iv) *Determination of purity of conjugate*

In order to determine whether the conjugation of glycine with cholic acid had been successful, samples of the resultant conjugation mixture (20µl) were run on TLC alongside standard solutions of glycocholic acid, cholic acid (both 20µl, 10mg ml^{-1} methanol), and glycine (20µl, 10mg ml^{-1} methanol/water (50/50)). TLC was run for 2 hours, with the mobile phase as described previously (see Fig. 2-4). The presence of compounds were detected by staining the dried plate with iodine.

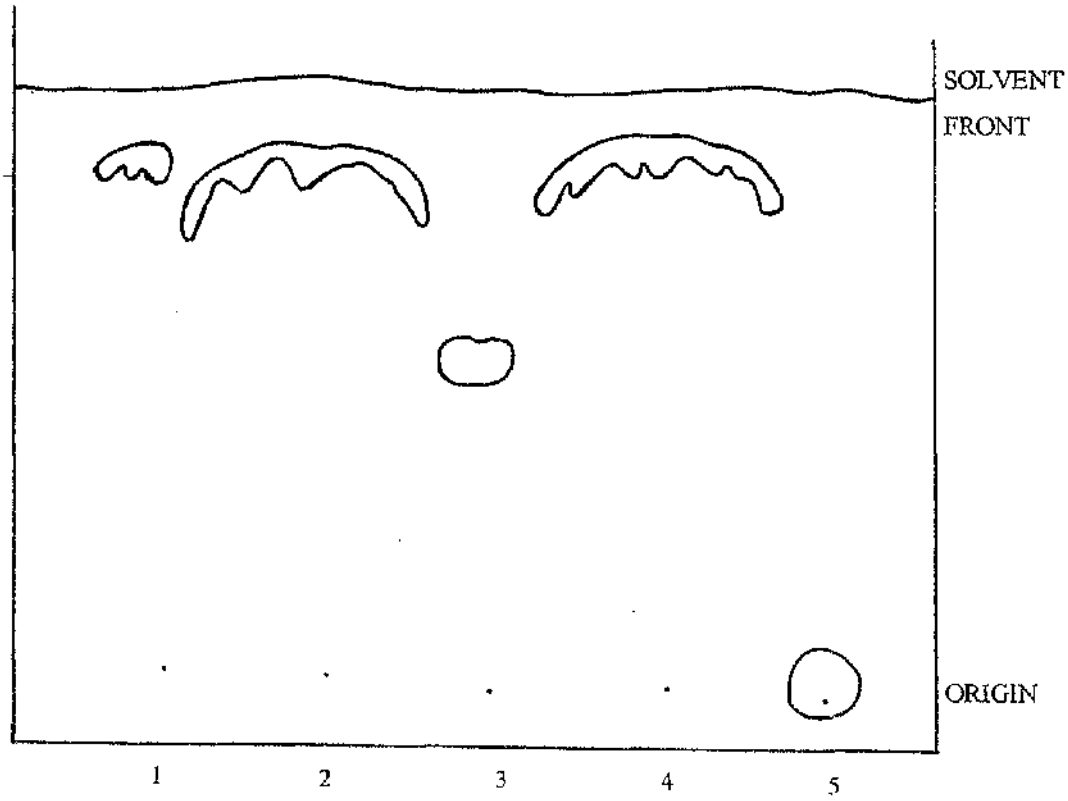


Fig 2-4: Thin layer chromatography of: (1) cholic acid (10mg ml^{-1}) (2) con 1 (3) glycocholic acid (10mg ml^{-1}) (4) con 2 and (5) glycine (10mg ml^{-1}), (all 20µl spots) on Silica Gel G layer, developed with butanol-glacial acetic acid-water (120+10+10) for 2 hours, and detected with iodine crystals. Exact tracing of developed TLC plate.

Figure 2-4 demonstrates the outcome of such a procedure. There was nothing to indicate that glycine had conjugated with cholic acid, since a spot with an Rf value corresponding to glycocholic acid was not obtained. Furthermore, there was no

evidence of any free glycine present in the two conjugation mixtures (con 1 and 2), suggesting that glycine had, therefore, not taken part in the conjugation reaction. Thus, it would appear that glycine-cholic acid conjugation had not been successful.

2.3.4 Tetragastrin-cholic acid conjugation

Since the conjugation of glycine with cholic acid was unsuccessful, presumably as a consequence of the insolubility of glycine in dimethyl formamide, it was decided to attempt the conjugation of tetragastrin (the C-terminal tetrapeptide amide sequence (Trp-Met-Asp-Phe amide) of gastrin. This was considered feasible since tetragastrin is more lipophilic and, therefore, less polar than glycine and was found to be completely soluble in dimethyl formamide. The structure of tetragastrin is shown in Figure 2-5.

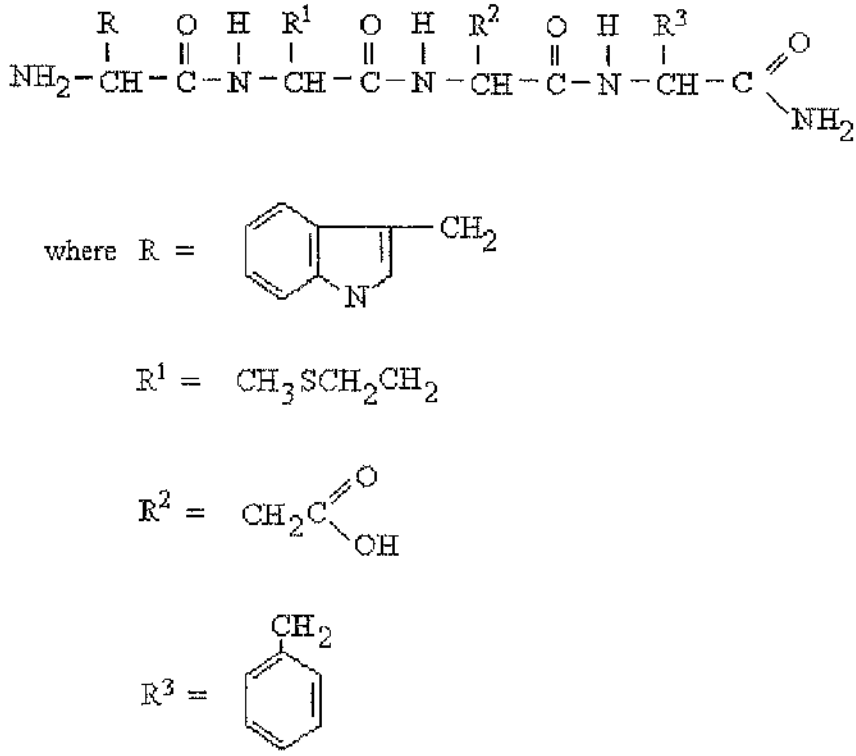


Fig. 2-5: Chemical structure of tetragastrin, where R is tryptophan; R^1 is methionine; R^2 is aspartate and R^3 is phenylalanine.

2.3.4(i) *Thin layer chromatography*

First, samples of cholic acid (10mg ml^{-1} methanol) and tetragastrin (5mg ml^{-1} methanol/water (50/50)) were run on TLC for a duration of 2 hours (Fig. 2-6), the mobile phase being equivalent to that described previously. This would enable a comparison with the results of TLC for the tetragastrin-cholic acid conjugate, once this had been performed.

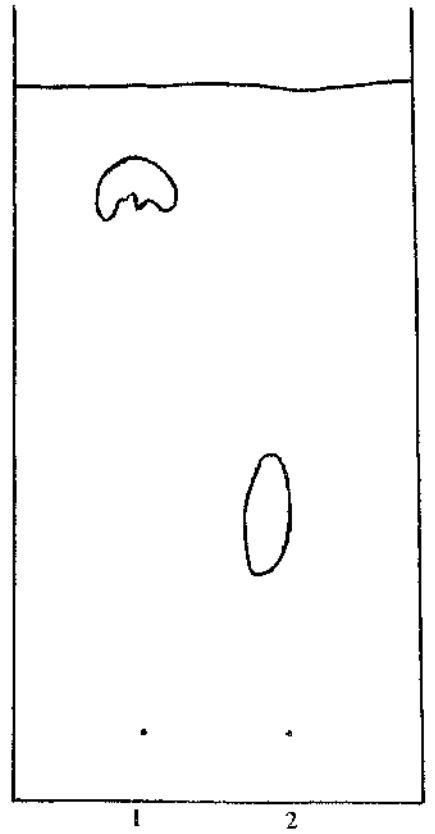


Fig. 2-6: Thin layer chromatography of: (1) cholic acid (10mg ml^{-1}) and (2) tetragastrin (5mg ml^{-1}) (both 20µl spots) on Silica Gel G layer, developed with butanol-glacial acetic acid-water (120+10+10) for 2 hours, and detected with iodine crystals. Exact tracing of developed TLC plate.

The Rf value of cholic acid was 0.855, a value very much in agreement with earlier work. Tetragastrin had a lower Rf value of 0.344. It was decided, by manipulation of the components of the mobile phase, to attempt to increase further the separation between the Rf values of cholic acid and tetragastrin. This would make identification of the tetragastrin-cholic acid conjugate easier. Butanol-glacial acetic acid-water (120 + 5 + 5) gave a more satisfactory result (Fig. 2-7) in that the Rf value for tetragastrin was reduced to 0.213, whereas the Rf value for cholic acid was relatively unchanged

(Rf = 0.825). This was the chemical composition of the mobile phase to be used in all subsequent procedures.

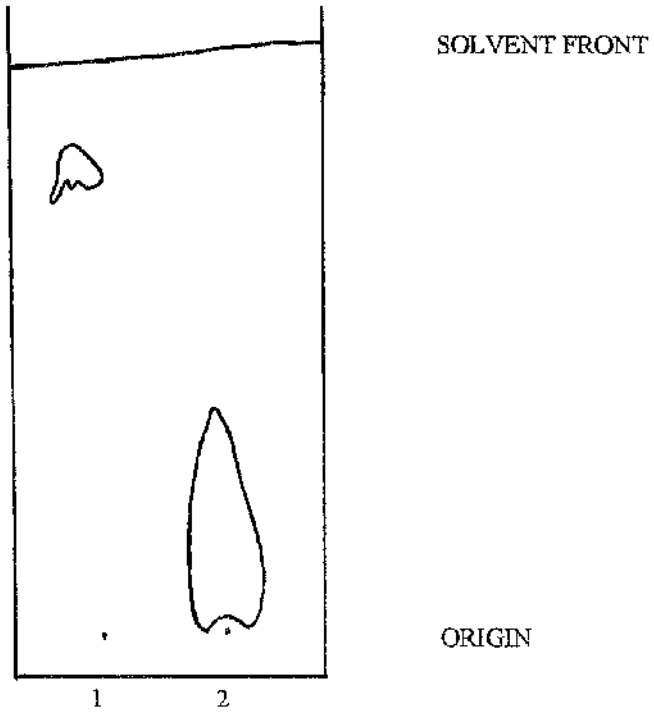
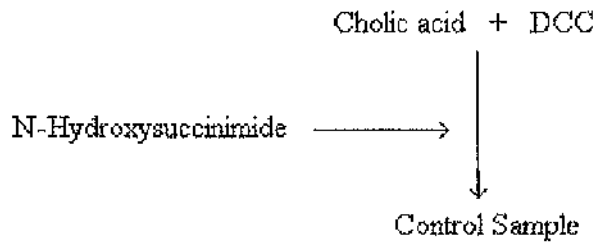


Fig. 2-7: Thin layer chromatography of: (1) cholic acid (10mg ml^{-1}) (2) tetragastrin (5mg ml^{-1}) (both 20µl spots) on Silica Gel G layer, developed with butanol-glacial acetic acid-water (120+5+5) for 2 hours, and detected with iodine crystals. Exact tracing of developed TLC plate.

2.3.4(ii) *The conjugation procedure*

The procedure for tetragastrin-cholic acid conjugation was carried out as follows: as before, each individual component of the conjugation reaction was dissolved in dimethyl formamide to give solution concentrations of 100mM. Equal volumes of cholic acid and DCC solution were mixed together, and to this was added an equal volume of N-hydroxysuccinimide solution. Without delay, the same volume of tetragastrin solution was added, and the resultant solution shaken vigorously. For ease of determination of the success of conjugation, a control solution was also made up, whereby all the components of the conjugation reaction were added, as described previously, with the exception of tetragastrin. Figure 2-8 summarises the steps taken to produce a control sample and a conjugation sample.

Control Sample Procedure



Conjugation Sample Procedure

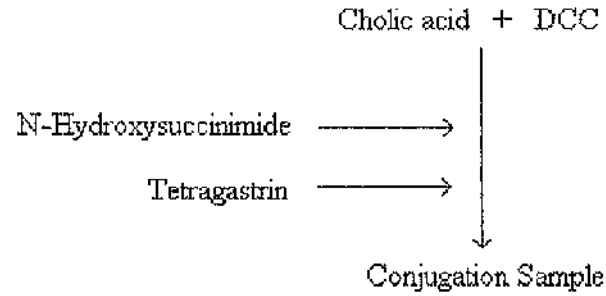


Fig.2-8: Diagram to illustrate the steps taken to produce a control and a conjugation sample

2.3.4(iii) *Determination of purity of conjugate*

TLC was used to determine whether the conjugation of tetragastrin with cholic acid had been successful, by applying spots of the conjugation and control sample on the chromatogram, alongside spots of cholic acid (100mM) and tetragastrin (100mM). In this case, 5µl samples were spotted in order to prevent the spread of each compound over an unsuitably large area. The method of TLC was carried out as described previously. Detection with iodine (Fig. 2-9) demonstrated that the tetrapeptide stained as a thin, extended streak, suggesting that it may not be as pure as was originally thought. The lack of staining in the centre of the conjugation sample implied that there may be some compound present which is not being detected with iodine. It was difficult to draw any conclusions about the success of the conjugation using the results from this chromatogram.

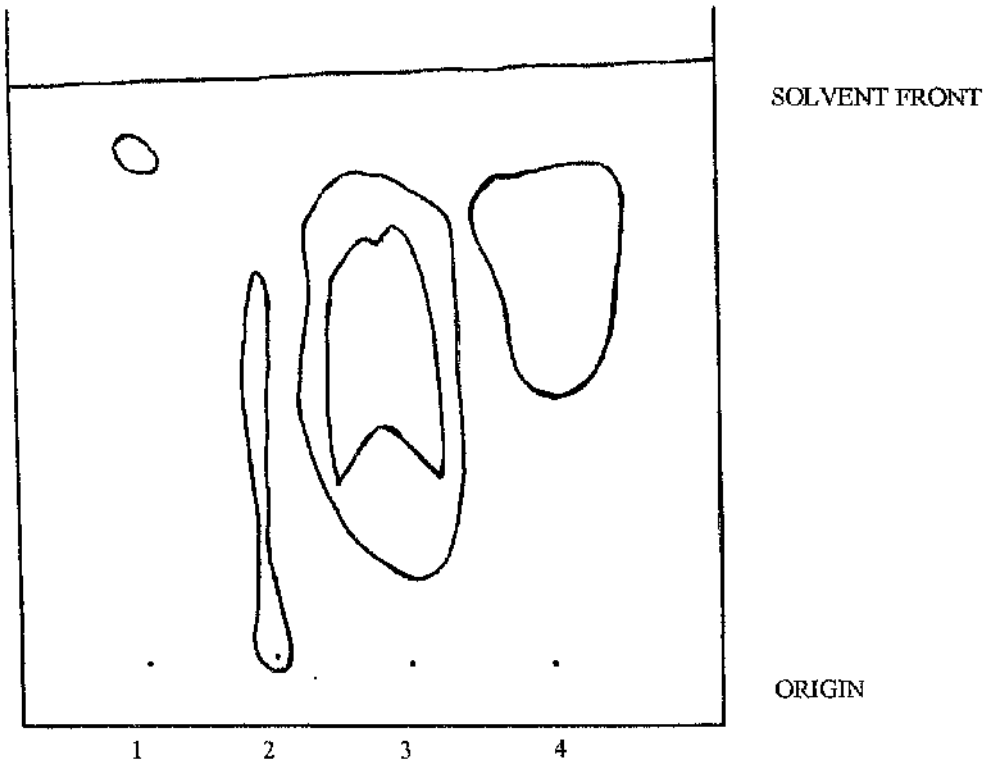


Fig. 2-9: Thin layer chromatography of: (1) cholic acid (100mM) (2) tetragastrin (100mM) (3) conjugation sample (4) control sample (all 5µl spots) on Silica Gel G layer, developed with butanol-glacial acetic acid-water (120+5+5) for 2 hours, and detected with iodine crystals. Exact tracing of developed TLC plate.

2.3.4(iv) *Partition of the conjugation sample*

It was decided to run TLC of a chromatographic plate spotted with the individual components of the conjugation mixture, in addition to spotting of the control sample, in an attempt to simplify the interpretation of the resultant plate. Furthermore, we added equal volumes of water and chloroform (1.0ml) to what was remaining of the conjugation sample from the previous reaction. This served to partition the components within the conjugation sample, that is, those that were water soluble would remain in the aqueous fraction (top layer), whereas the lipid soluble components would be found in the chloroform fraction (bottom layer). This, therefore, provided a means of further separating the components within the conjugation sample, with the hope that the complex nature of the sample would be reduced. A sample from both the aqueous and chloroform fraction was applied to the origin of the chromatogram.

The sample size of each of the spotted samples was adjusted in order to enable a more direct comparison. The individual components of the conjugation sample were applied as 2.5µl spots; whereas the spot size of the control sample was 7.5µl, and that of the conjugation sample was 10µl. These volumes were decided upon since the concentration of each of the individual components in the control sample and in the conjugation sample would be 3 and 4 times more dilute, respectively, than in the solutions composed solely of either cholic acid, DCC, N-hydroxysuccinimide or tetragastrin. Therefore, the spotted samples would be equivalent in quantity of each component.

TLC was carried out as described earlier, and iodine crystals were employed as the means of detection (Fig. 2-10). Both the sample taken from the aqueous fraction of the conjugation sample, and tetragastrin appeared as very similar thin, extended streaks, making it difficult to determine whether conjugation had been successful.

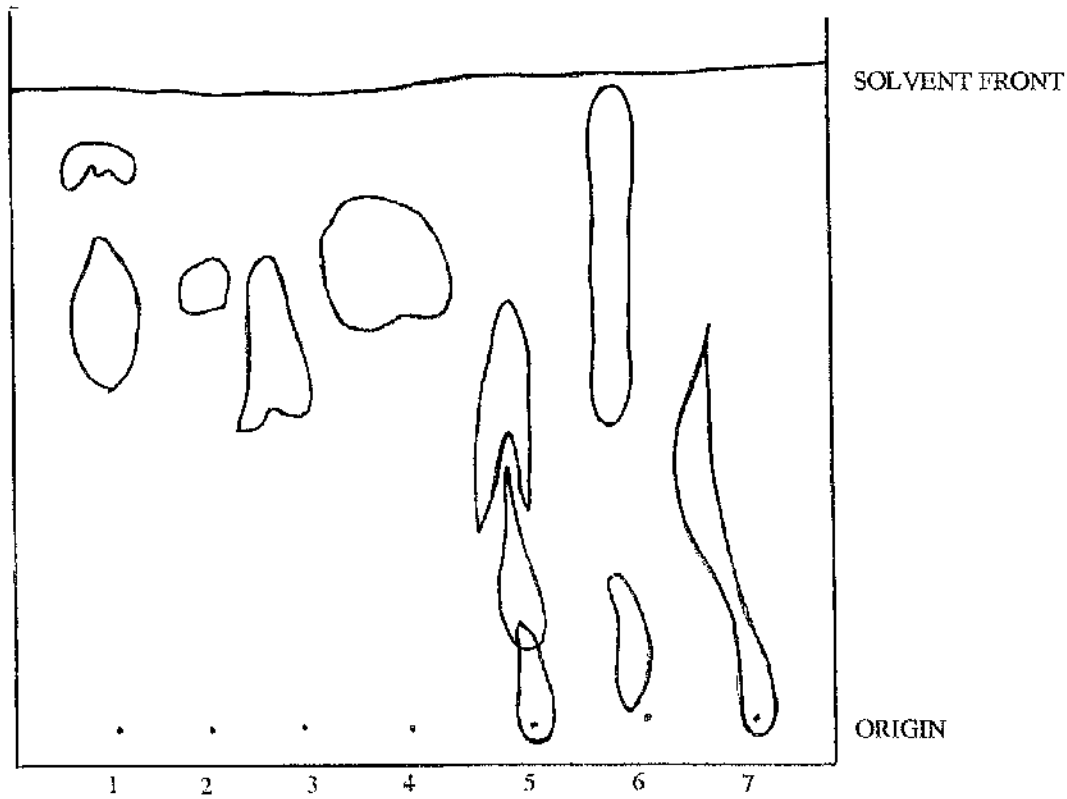


Fig. 2-10: Thin layer chromatography of: (1) cholic acid (100mM); (2) DCC (100mM); (3) N-hydroxysuccinimide (100mM) (all 2.5µl spots); (4) control sample (7.5µl); (5) aqueous fraction of conjugation sample (10µl); (6) chloroform fraction of conjugation sample (10µl); (7) tetragastrin (100mM) (2.5µl) on Silica Gel G layer, developed with butanol-glacial acetic acid-water (120+5+5) for 2 hours, and detected with iodine crystals. Exact tracing of developed TLC plate.

2.3.4(v) *Ninhydrin as a visualising reagent*

It was, therefore, decided to stain the same chromatogram with ninhydrin (1, 2, 3-Indantrione), since this is a reagent which stains proline and hydroxyproline yellow, and all other α-amino acids violet (Touchstone and Dobbins, 1978), and would establish whether free peptides were present within the conjugation sample and the tetragastrin sample. In order to do this, ninhydrin crystals (0.5g) were dissolved in ethanol (100ml), and the resultant solution sprayed evenly onto the plate. The chromatogram was then heated using a hairdryer set on the highest heat setting for approximately 30 minutes.

Figure 2-11 demonstrates the outcome of such a procedure. As was to be expected, there were no free peptides present in the control sample nor within the individual components of the control sample. The aqueous fraction of the conjugation sample and tetragastrin sample, when stained with ninhydrin, were very similar, indicating the presence of amino acids (or free peptides) over an area very close to the origin.

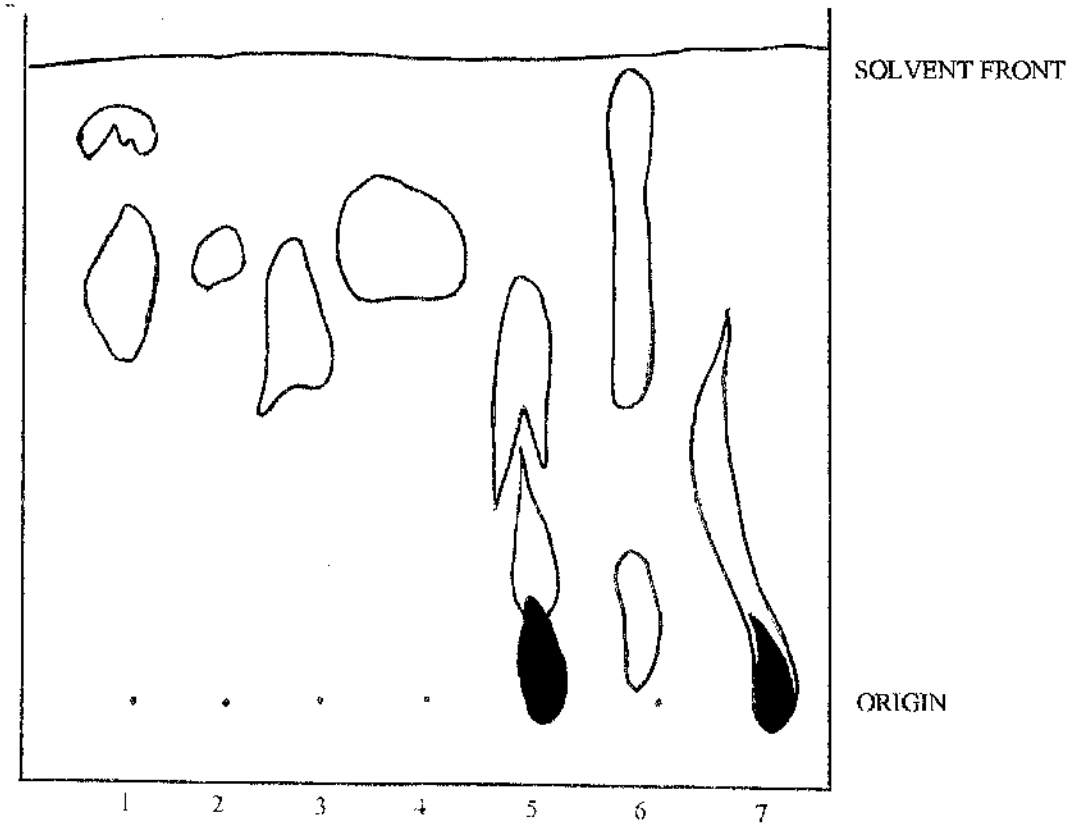


Fig. 2-11: Thin layer chromatography of: (1) cholic acid (100mM); (2) DCC (100mM); (3) N-hydroxysuccinimide (100mM) (all 2.5μl spots); (4) control sample (7.5μl); (5) aqueous fraction of conjugation sample (10μl); (6) chloroform fraction of conjugation sample (10μl); (7) tetragastrin (100mM) (2.5μl) on Silica Gel G layer, developed with butanol-glacial acetic acid-water (120+5+5) for 2 hours, and detected with iodine crystals (unshaded) and ninhydrin (shaded). Exact tracing of developed TLC plate.

Nevertheless, any indication that conjugation between tetragastrin and cholic acid had been achieved was difficult to establish. Furthermore, the long streak of the tetragastrin sample, stained by iodine but unstained by ninhydrin, supported the idea that the tetrapeptide was not as pure as was hoped, and further, implied that the impurity was not an amino acid(s).

2.3.4(vi) *Contribution of dimethyl formamide*

In order to determine whether the presence of dimethyl formamide contributed to the apparent contamination of tetragastrin, dimethyl formamide alone was spotted alongside tetragastrin (25mM, made up in alcohol/water (50/50)), and the aqueous fraction of the conjugation sample (Fig. 2-12).

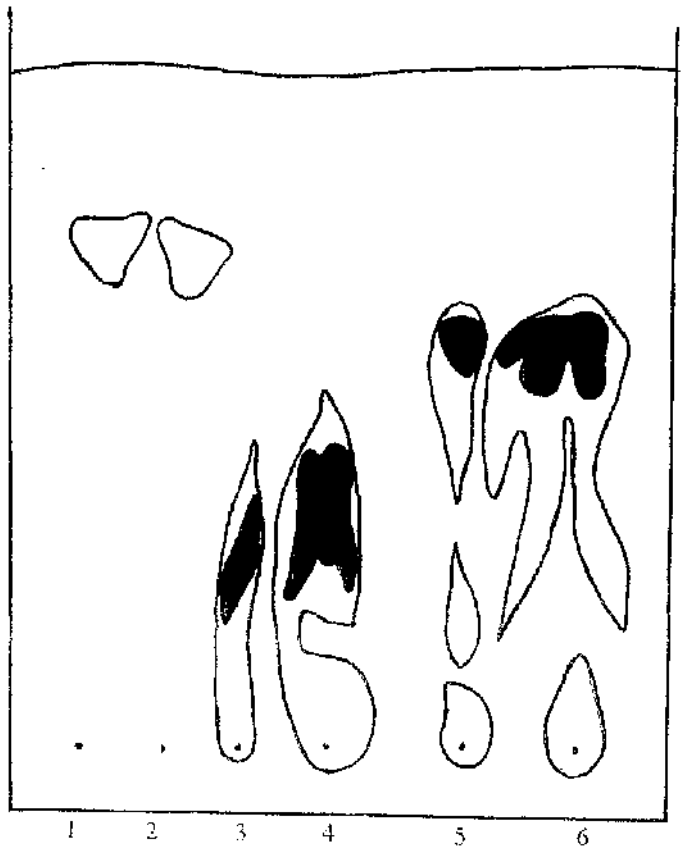


Fig. 2-12: Thin layer chromatography of: (1) dimethyl formamide (2.5μl): (2) dimethyl formamide (7.5μl); (3) tetragastrin (25mM, 10μl); (4) tetragastrin (25mM, 30μl); (5) aqueous fraction of conjugation sample (10μl); (6) aqueous fraction of conjugation sample (30μl) on Silica Gel G layer, developed with butanol-glacial acetic acid-water (120+5+5) for 2 hours, and detected with iodine crystals (unshaded) and ninhydrin (shaded). Exact tracing of developed TLC plate.

The chromatographic procedure was as described previously, and after 2 hours, visualisation of the samples was carried out by first staining with iodine, and then employing ninhydrin, as described earlier. The stained areas provided evidence to suggest that dimethyl formamide was not a contributory factor in the contamination of tetragastrin, and supported the premise that the tetrapeptide was contaminated with an other substance(s). The stained areas of this plate were quite different from the previous (Fig. 2-11) in that the ninhydrin-stained areas of tetragastrin were central within the iodine stain, rather than close to the origin, and the ninhydrin-stained areas of the conjugation sample were found towards the top of the iodine-stained area. However, this plate was somewhat unsatisfactory since the iodine staining had left a pinkish tinge, making the ninhydrin stained areas difficult to establish.

In order to avoid such a problem, TLC of the individual components of the conjugation sample was carried out alongside TLC of the aqueous fraction of the conjugation sample, as described earlier; however, the iodine staining process was omitted, and the plate stained using ninhydrin (Fig. 2-13). Tetragastrin was detected as an extended streak similar to iodine-stained tetragastrin (Figs. 2-11 and 2-12). The aqueous fraction of the conjugation sample was detected in two areas of the plate: at the origin, and close to the solvent front, at a region not dissimilar to the tip of the tetragastrin stained band. However, it was very difficult to conclude whether conjugation had been successful, although the absence of staining in the central part of the conjugation sample band suggests it may have been.

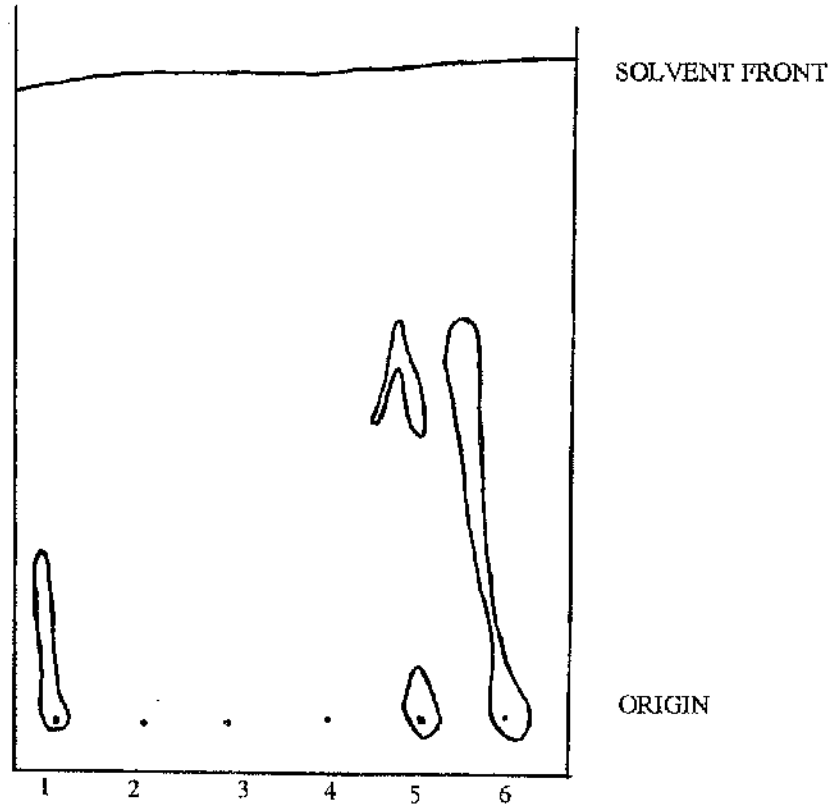


Fig. 2-13: Thin layer chromatography of: (1) tetragastrin (25mM, made up in methanol/water (50/50)) (10µl); (2) cholic acid (100mM) (2.5µl); (3) DCC (100mM) (2.5µl); (4) N-hydroxysuccinimide (100mM) (2.5µl); (5) aqueous fraction of conjugation sample (10µl); (6) tetragastrin (100mM) (2.5µl) on Silica Gel G layer, developed with butanol-glacial acetic acid-water (120+5+5) for 2 hours, and detected with ninhydrin. Exact tracing of developed TLC plate.

2.3.4(vii) *Determination of purity of tetragastrin*

It was of importance to establish the purity of the tetrapeptide prior to any further attempts at conjugation, since any contamination would influence the success of such a venture.

This was initially carried out using high performance liquid chromatography (HPLC), performed by a Shimadzu LC10 chromatographer with diode ray detector. Analysis of a 5µg injection of tetragastrin (Trp-Met-Asp-Phe amide) was carried out at a gradient of 20-40% in 0.05% Trifluoracetic acid (TFA)/H_2O and 0.05% TFA/Methyl cyanide (MeCN), and detected using UV monitoring at 215nm and 280nm. Generally, HPLC analysis at 215nm is the traditionally selected option, and 280nm discloses compounds containing aromatic groups, such as phenylalanine and tryptophan. For this reason, the chromatogram at 280nm provides the best guide for

assessment of our sample. Furthermore, as time increases (horizontal axis), the polarity of the compounds diminishes.

A main peak appearing as a doublet was present (Fig. 2-14). This suggested that there were two major components of the tetrapeptide, and may be due to a number of factors, for example, racemization of one amino acid, the presence of a free acid with an amide, oxidation of methionine, or rearrangement of β-aspartimide.

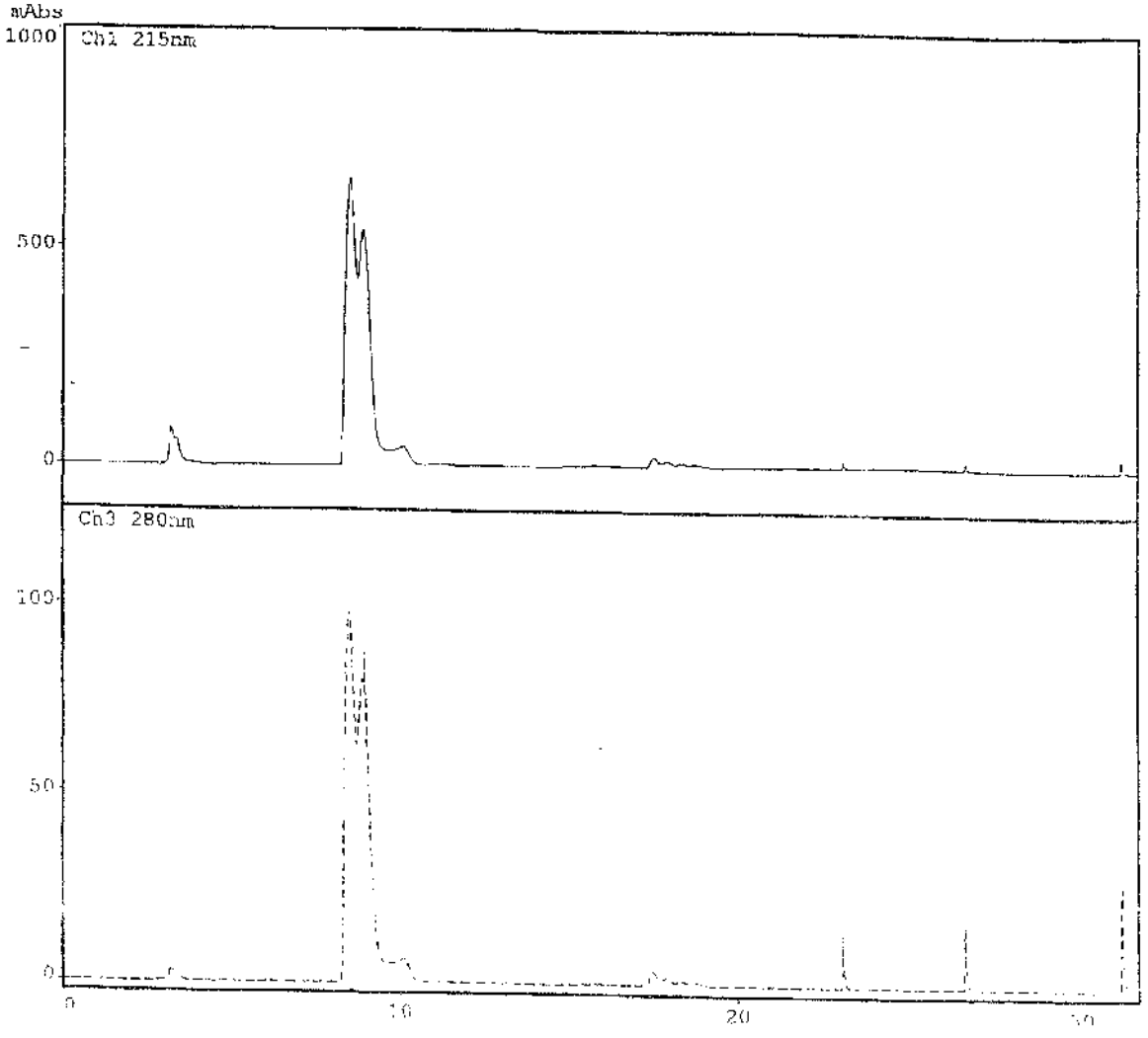


Fig. 2-14: HPLC of Trp-Met-Asp-Phe amide (tetragastrin) (5μg) at a gradient of 20-40% in 0.05% TFA/H_2O and 0.05% TFA/MeCN. Detection using UV monitoring at 215nm (upper trace) and 280nm (lower trace). Axes indicate relative abundance against time of elution in min.

In order to obtain a more precise understanding of the composition of tetragastrin, a sample of the tetrapeptide was analysed using electrospray mass spectrometry in positive ion mode. This was performed on a VG BioTech platform single quadrupole mass spectrometer fitted with a pneumatically-assisted electrospray source, and controlled via the VG Masslynx software. The carrier solvent was 1:1 (v/v) acetonitrile:water with formic acid added to give a final concentration of 0.2% (v/v). The mass spectrum (Fig. 2-15) demonstrated the presence of two ions. (The actual mass of the detected molecules is equivalent to the value on the horizontal scale (Da/e) minus one, taking into account an extra proton (H^+) added in the ionisation of the compound.) The larger peak shows an ion at 597 Da/e, set to an abundance of 100%. An ion at 653 Da/e, with a relative abundance of 8%, was also found to be present. These results are indicative of the relatively uncontaminated nature of the tetragastrin sample, though they do reveal the presence of an additional compound of molecular weight 652Da, the identity of which is unknown, but which may have arisen in the process of manufacture (see Section 2.3.7).

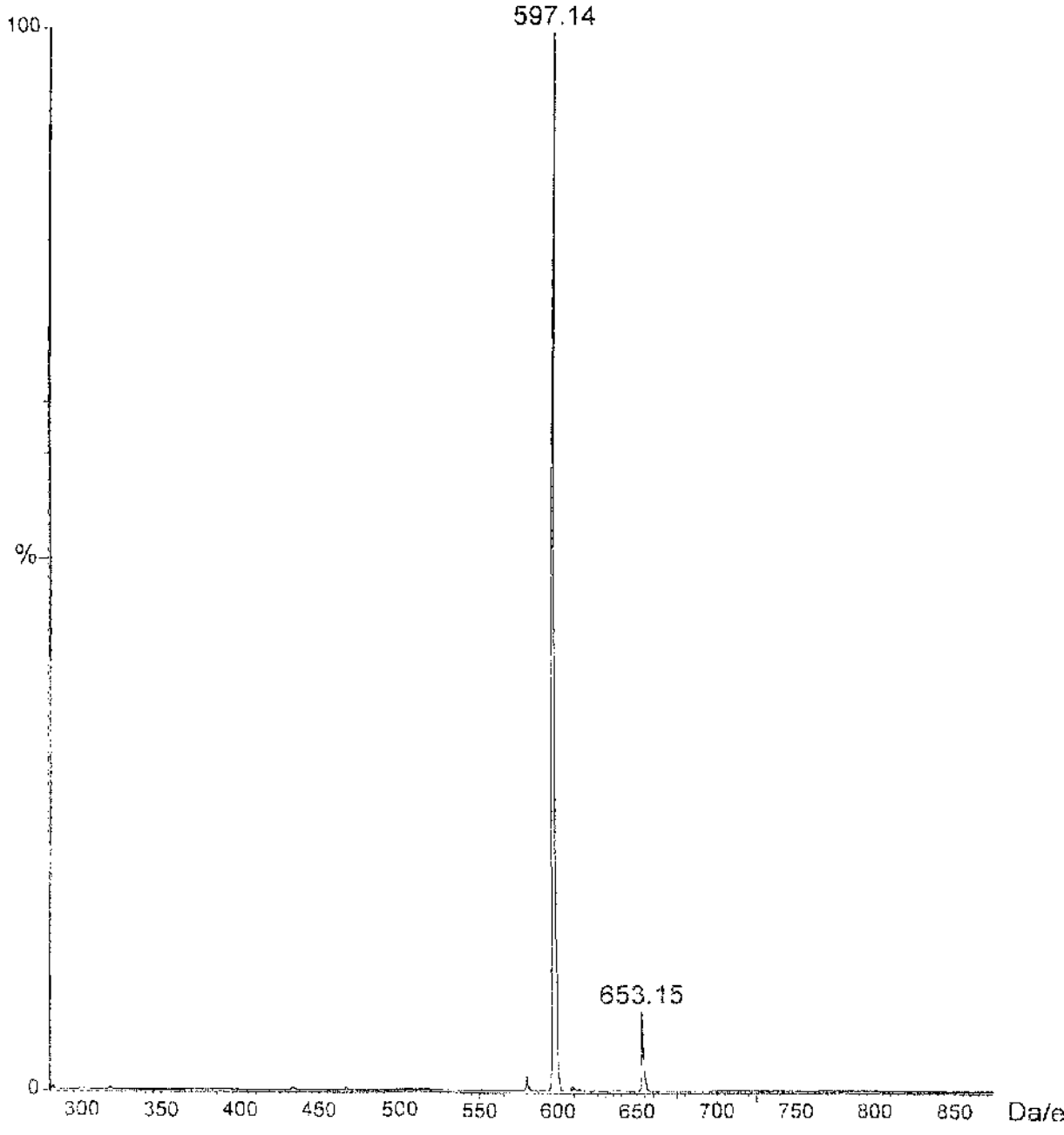


Fig. 2-15: Mass spectrum of Trp-Met-Asp-Phe amide (tetragastrin) obtained by electrospray mass spectrometry. Spectrum is plot of relative abundance, with the largest peak assigned 100%, against the molecular weight.

2.3.4(viii) *Visualisation using Ehrlich's reagent*

We decided, therefore, to aim for a more specific stain when carrying out TLC as a procedure to determine the success of tetragastrin-cholic acid conjugation.

Ehrlich's reagent (4-dimethylaminobenzaldehyde-HCl) has been shown to be specific for the detection of citrulline, urea, tryptamine and tryptophan (Stahl, 1965). In order to verify this, L-tryptophan (10mg ml^{-1} 0.1M HCl) was applied to a silica gel G plate as 10, 20 and 30µl spots and allowed to dry, before a fine mist of Ehrlich's reagent (1%) was sprayed evenly over the plate. On drying, tryptophan was found to stain bright yellow.

TLC of the individual components of the conjugation sample, the aqueous and chloroform fractions of the partitioned conjugation sample, and the control sample was run as described earlier. Ehrlich's reagent was employed as the visualisation reagent, rather than iodine or ninhydrin (Fig. 2-16).

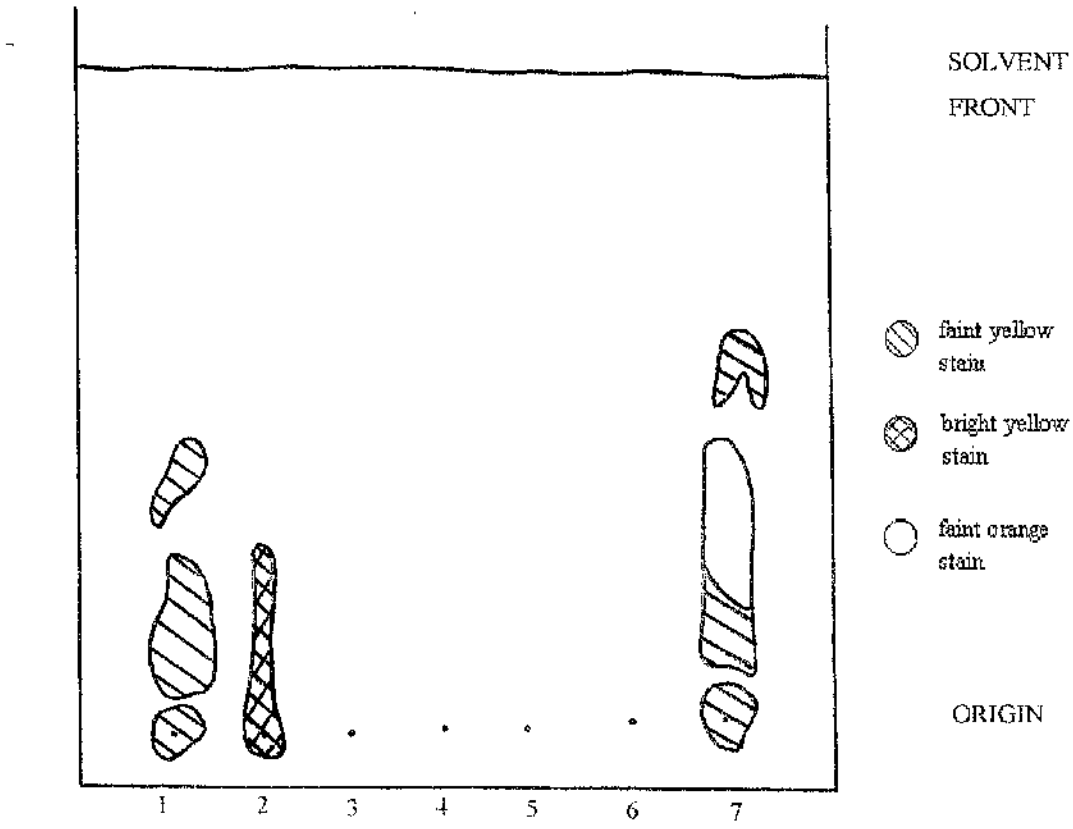


Fig. 2-16: Thin layer chromatography of: (1) aqueous fraction of conjugation sample (10µl); (2) tetragastrin (100mM) (2.5µl); (3) DCC (100mM) (2.5µl); (4) control sample (7.5µl); (5) cholic acid (100mM) (2.5µl); (6) N-hydroxysuccinimide (100mM) (2.5µl); (7) chloroform fraction of conjugation sample (10µl) on Silica Gel G layer, developed with butanol-glacial acetic acid-water (120÷5÷5) for 2 hours, and detected with Ehrlich's reagent. Exact tracing of developed TLC plate.

This step was of considerable importance since it was anticipated that DCC, present in both the conjugation sample and control sample, may breakdown into urea-like components for which Ehrlich's reagent would stain positively. However, the spotted samples of DCC, cholic acid, N-hydroxysuccinimide and the control sample were unstained when the plate was sprayed with Ehrlich's reagent. The tetrapeptide appeared as a bright yellow, extended spot, presumably as a result of the presence of tryptophan, and had a very similar appearance to the aqueous fraction of the conjugation sample, although the latter stained less brightly. The chloroform fraction of the conjugation sample stained as two distinct streaks. One of these was very similar to the stain of both tetragastrin and the aqueous fraction of the conjugation sample, but the second was displaced nearer to the solvent front. It was considered possible that this could be the cholic acid-tetragastrin conjugate.

2.3.4(ix) *Elution of chloroform fraction of conjugation sample*

Elution is the removal of a solute from the sorbent by the passage of a suitable solvent. Such a process is useful in the isolation of a solute, enabling further analysis, for example, mass spectrometry, to be carried out.

Since it was anticipated that the chloroform fraction of the conjugation sample may contain the tetragastrin-cholic acid conjugate, we wished to carry out elution of this fraction, in order to clarify this. A sample of the chloroform fraction of the partitioned conjugation sample (10µl) was applied at either side of a wide streak of the chloroform fraction (Fig. 2-17), and TLC was run as before.

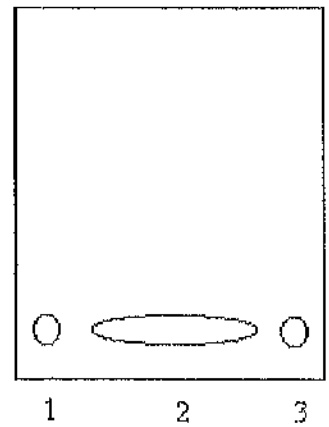


Fig. 2-17: Sketch of silica gel G plate prior to running TLC where (1) and (3) are spots of chloroform fractions of conjugation sample (10µl), and (2) is a streak of chloroform fraction of conjugation sample.

Strips, consisting of the two vertical margins of the plate where the 10µl spots were applied, were cut away from the central part of the plate (where the streak had been applied), and stained with Ehrlich's reagent. Two distinct bands were noted on each strip. Alignment of the stained strips with the unstained central plate allowed us to predict where the central streak would have been displaced to. Using pencil, these two regions were marked out on the central plate, and were referred to as the top section and bottom section (Fig. 2-18).

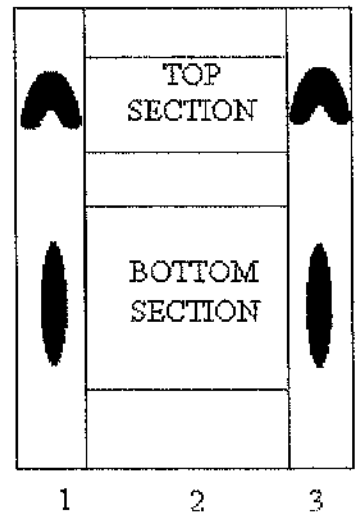


Fig. 2-18: Sketch of silica gel G plate after running TLC of (1) and (3) chloroform fraction of conjugation sample (10µl), and (2) streak of chloroform fraction of conjugation sample, using butanol-glacial acetic acid-water (120+5+5) as mobile phase, and staining strips (1) and (3) with Ehrlich's reagent. Top section and bottom section bands were marked out on the central plate.

The silica from the bottom section band was scraped from the central plate, and poured into a scintered glass funnel. This was repeated, using another scintered glass funnel, with the silica from the top section band. These will be denoted as bottom section silica, and top section silica, respectively. The top section silica was eluted using a solvent composed of 95% butanol: 5% glacial acetic acid. Since cholic acid has a high Rf value with this solvent, running near to the solvent front in a similar way to the top staining band (top section silica), this solvent was considered suitable for the removal of the top staining compound from the silica. The bottom section silica was eluted in the same way as the top section, except that this time the solvent chosen was 80% glacial acetic acid: 20% water, in order to separate the bottom staining band

from the silica. In both cases, the solvent running through the silica was collected in pre-weighed vessels. Figure 2-19 illustrates the technique of removal of compounds from the silica gel plates.

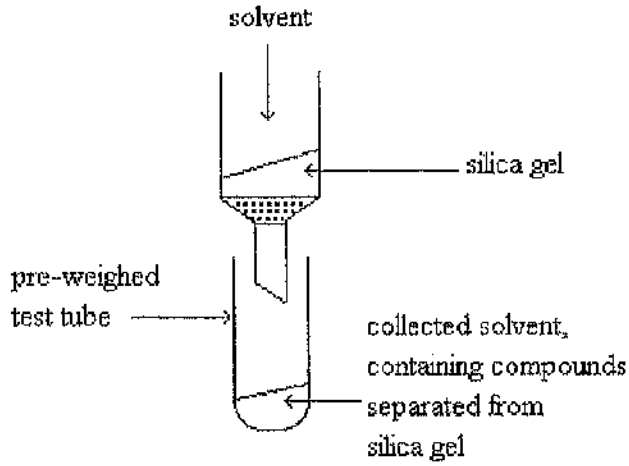


Fig. 2-19: Experimental set-up for elution of chloroform fraction of conjugation sample.

The next step was to blow off the solvent collected in the pre-weighed test tubes, in order to leave a solid deposit containing the compounds present in the top and bottom section silica. In order to get rid of the 95% butanol: 5% glacial acetic acid, the contents of the test tube were warmed in a water bath at 40°C, whilst passing a stream of nitrogen over the top of the test tube. The 80% glacial acetic acid: 20% water was removed by placing the test tube in a dessicator containing NaOH pellets, and attaching it to a water pump. The water pump set up a vacuum in the dessicator, helping to evaporate glacial acetic acid (Fig 2-20). In both cases, the solvent was

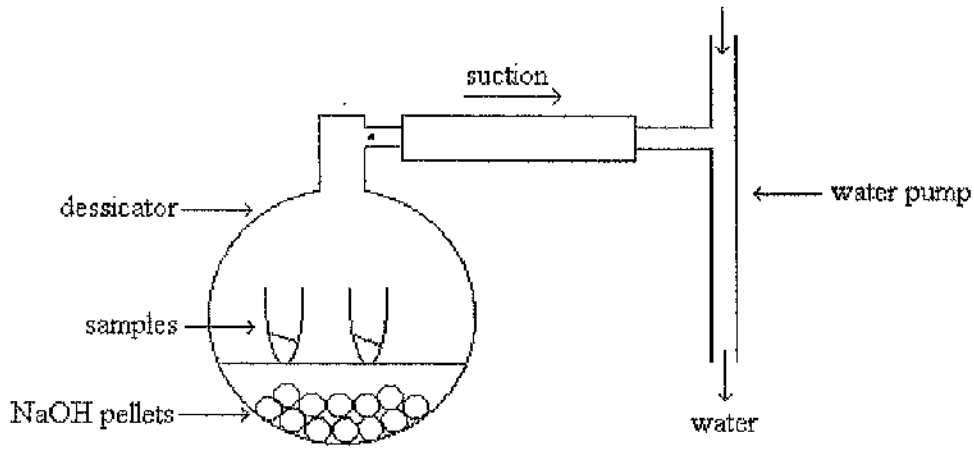


Fig. 2-20: Diagram of apparatus used to evaporate glacial acetic acid:water.

removed successfully, leaving a white sediment in the test tubes. Dimethyl formamide was added to the sediment in both cases, to give a final solution of approximately 10mg ml^{-1}.

The sediment recovered from elution of each of the top and bottom section silica were applied in duplicate as 10µl spots at the origin of a chromatographic plate, and TLC was developed using butanol-glacial acetic acid-water (120 + 5 + 5) as the mobile phase. After 2 hours, the plate was removed, dried, and bisected vertically so that both halves of the plate, each with eluted top and bottom section compounds could be stained using different reagents. Iodine (universal stain) was used to visualise the displacement of compounds on one plate, whereas Ehrlich's reagent (stains positively for citrulline, urea, tryptamine and tryptophan) was employed as the visualisation reagent for the other plate.

The iodine stained plate demonstrated similar displacements of the top and bottom section eluted compounds, close to the solvent front, with Rf values very much in agreement with that of cholic acid (chromatogram not shown but similar to Fig. 2-21). Ehrlich's reagent did not stain positively, thereby suggesting that neither the tetrapeptide nor its cholic acid conjugate was present (chromatogram not shown).

2.3.4(x) *Thin layer chromatography of eluted samples*

TLC was carried out again in a similar way to that just described, although this time an additional sample of cholic acid (2.5µl, 100mM) was applied, in order to provide a standard to compare with the eluted samples. The iodine stained plate provided evidence to suggest that cholic acid was present in the eluted samples from both the top and bottom sections, since stained bands similar to that of cholic acid were evident (Fig 2-21). As was shown by the previous plate, there were no positively staining compounds when Ehrlich's reagent was employed as the visual reagent (chromatogram not shown).

The entire elution procedure was repeated once more, as has already been described. However, this time double the volume of the chosen solvent for elution (that is, either 95% butanol: 5% glacial acetic acid, or 80% glacial acetic acid: 20% water) was used, in order to improve the likelihood of removing the compounds from the silica. However, TLC demonstrated that the eluted top and bottom section silica appeared to contain the same compound(s), and furthermore, had a very similar Rf value to cholic

acid. There were no Ehrlich positive staining compounds, suggesting that neither eluted samples contained either tetragastrin or the tetragastrin-cholic acid conjugate (chromatogram not shown).

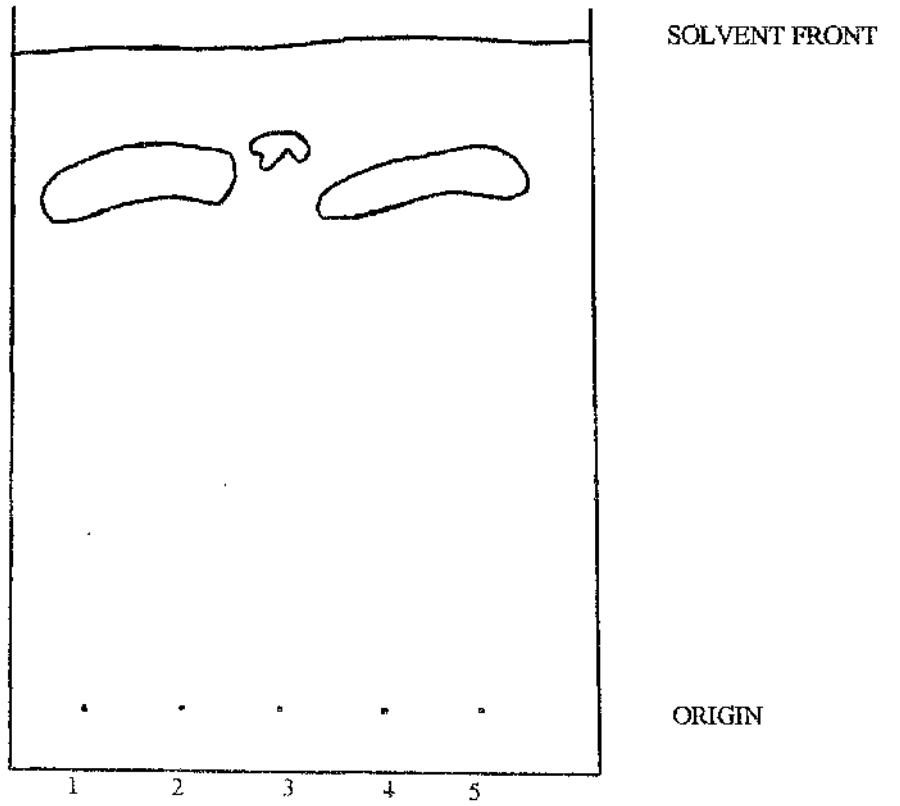


Fig. 2-21: Thin layer chromatography of: (1) and (2) eluted bottom section silica (~8mg ml^{-1}, 10µl); 3) cholic acid (100mM, 2.5µl); (4) and (5) eluted top section silica (~8mg ml^{-1}, 10µl) on Silica Gel G layer, developed with butanol-glacial acetic acid-water (120+5+5) for 2 hours, and detected with iodine. (Rf of eluted bottom section silica: 0.802; Rf of eluted top section silica: 0.813; Rf of cholic acid: 0.855.) Exact tracing of developed TLC plate.

2.3.4(xi) *Mass spectrometry of eluted samples*

The difficulties encountered in interpretation of the results of TLC indicated that a more definitive approach should be taken. It was decided to carry out analysis of the eluted top and bottom section silica by electrospray mass spectrometry (as described previously), a technique which determines the molecular weights of the components in a sample. For reference, the molecular weights of the individual components of the conjugation sample are listed in Table 2-1.

Table 2-1: Molecular weights of components of conjugation sample

COMPONENT	MOLECULAR WEIGHT
Cholic acid	408
DCC	206
N-Hydroxysuccinimide	115
Tetragastrin	596
Tetragastrin-cholic acid conjugate	*986*

However, as dimethyl formamide was not considered suitable for use in the mass spectrometer, a more volatile solvent was required. Therefore, the entire elution procedure of top and bottom section silica was carried out once again, and the recovered sediment from each region dissolved in 1:1 TFA (0.1%): acetonitrile, to give final solution concentrations of approximately 6mg ml^{-1}. Both samples underwent analysis in the mass spectrometer.

Mass spectrometry of both the top and bottom eluted sections demonstrated that ionisation of the samples produced a huge number of ions (Fig. 2-22). The presence of an ion at 413 Da/e, with an assigned abundance of 100%, occurred in both spectra. However, there were a considerable number of other ions ranging from between 200 and 1200 Da/e, of varying relative abundances, making analysis of the samples very difficult. The tetragastrin-cholic acid conjugate would be identifiable as an ion at approximately 987 Da/e, since the conjugation procedure involves a condensation reaction. So, for every molecule of the conjugate produced, a molecule of water (molecular mass of 18) would be lost from the two reactants. However, it was unlikely that the samples contained the tetrapeptide-cholic acid conjugate, since an ion at or close to 987 Da/e was not apparent from the spectrum obtained from either the top section or bottom section silica.

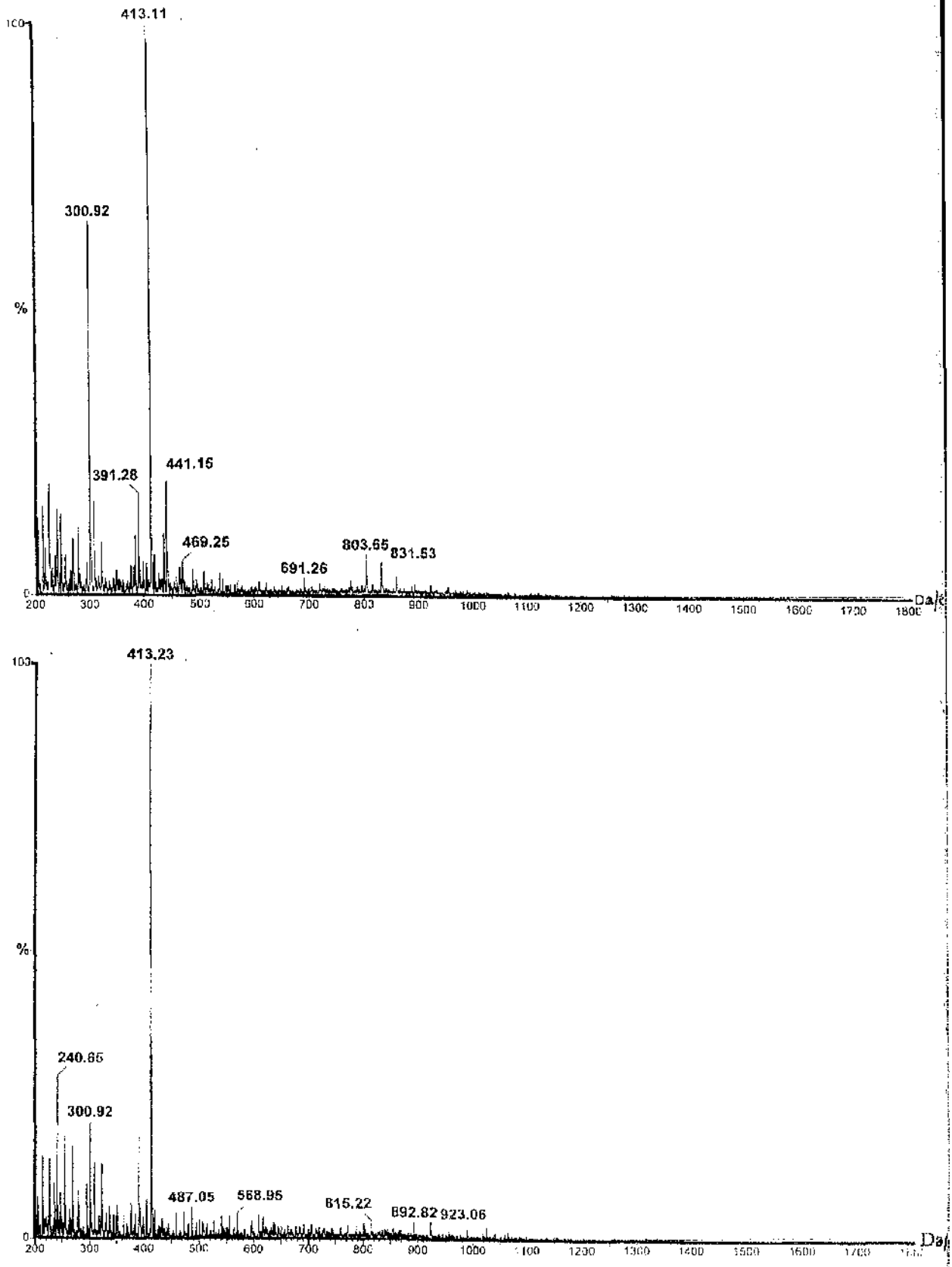


Fig: 2-22: Mass spectrum of eluted top section silica (upper trace) and eluted bottom section silica (lower trace), obtained by electrospray mass spectrometry. Spectrum is plot of relative abundance, with the largest peak assigned 100%, against the molecular weight.

2.3.4(xii) *Tetragastrin-cholic acid conjugation using higher concentration of reaction components*

It was therefore decided to attempt the conjugation of tetragastrin with cholic acid a further time, using solutions of the individual components with stronger concentrations (500mM rather than 100mM), in order to increase the yield. The actual conjugation procedure was as described previously (Fig. 2-8); however, rather than carrying out TLC of the conjugation sample, and subsequent elution of the silica gel, it was decided that electrospray mass spectrometry of the conjugation sample itself should be attempted. Since the final conjugation sample was dissolved in dimethyl formamide, a solvent not particularly suitable for use in the mass spectrometer, it was necessary to blow off this solvent using a stream of nitrogen directed over the sample. The remaining crystals were then dissolved in 1:1 TFA (0.1%):acetonitrile to give a final solution of approximately 20mg ml^{-1}.

Electrospray mass spectrometry of this sample produced a spectrum (Fig. 2-23) demonstrating an unidentifiable ion with an assigned abundance of 100% at 322 Da/e. The presence of an ion at 597 Da/e, considered to be tetragastrin, had a relative abundance of 30.5%. Ions at 579 and 615 Da/e, with relative abundances of 4 and 6%, respectively, may represent tetragastrin with a molecule of water removed or added, respectively. The presence of an N-hydroxysuccinimide derivative of the ion at 579 Da/e, with a relative abundance of 35%, was indicated by an ion at 693 Da/e. Two ions were present at 1005 and 1102 Da/e, both with relative abundances of 11%. The ion at 1005 Da/e suggested the presence of the product of the reaction between the ion at 615 Da/e and cholic acid, with the resultant loss of a water molecule. The ion at 1102 Da/e may have been the product of the reaction between cholic acid and the N-hydroxysuccinimide derivative of tetragastrin. However, there was no evidence to suggest the presence of the tetragastrin-cholic acid conjugate, since no ion was detected at or near 987 Da/e.

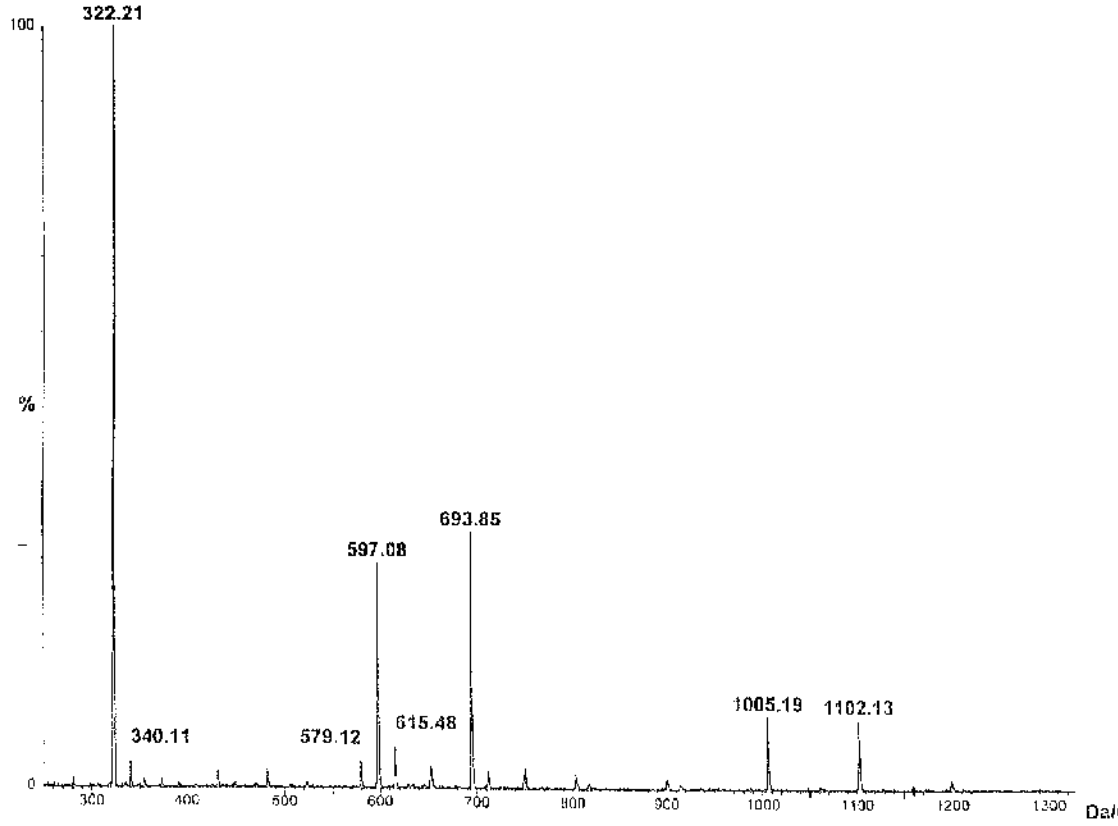


Fig. 2-23: Mass spectrum of conjugation sample, as obtained by electrospray mass spectrometry. Spectrum is plot of relative abundance, with the largest peak assigned 100%, against the molecular weight.

2.3.5 Tetragastrin-cholic acid conjugation - modified procedure

The unsuccessful conjugation of cholic acid with tetragastrin suggested that a number of modifications be made to the conjugation procedure as required.

2.3.5(i) *Purification and drying of reagents/solvents*

First, it was necessary to purify the DCC source prior to its use, since it was highly probable that some N-N'-Dicyclohexylurea (DCU), the hydrated DCC adduct, would have been formed on exposure of DCC to the atmosphere. The procedure for doing this is outlined overleaf:

- The top layer of the DCC was scraped off and disposed of.
- The vessel containing DCC was warmed slowly to a temperature of approximately 40°C (DCC has a melting point of between approximately 34 and 35 °C), and the resultant liquid poured into a screw-top bottle.
- DCC was dissolved in excess dichloromethane (DCM), a solvent in which DCC has high solubility. The sample was then stored in the fridge overnight in order to allow the solid precipitation of DCU to form as a white solid.
- DCU was filtered off, and the DCM removed from the sample by gentle heating in a water bath at 40°C under a stream of nitrogen. The purified DCC remaining was immediately transferred to a vacuum in order to prevent rehydration.

It was considered essential that the reagents involved be as dry as possible, since water would interact with the reactive intermediary of the conjugation reaction. This was achieved by placing samples of the reagents in the dessicator with attached water pump (Fig. 2-20) in order to create vacuum-like conditions, and allowing any water present to evaporate. The glassware to be used was also dried in an oven and placed in the dessicator until required, in order to prevent the reabsorption of water.
Acetonitrile was considered to be a more suitable solvent than dimethylformamide (DMF), since DMF is reported to encourage the formation of the N-acylurea side product, as well as increasing racemization in chiral carboxylic acids (Paquette, 1995). Acetonitrile is also easier to remove from the final product. However, it was discovered that cholic acid was highly insoluble in acetonitrile, and we therefore felt compelled to resort once again to DMF as the solvent. Since it was considered imperative that water should not contaminate the conjugation reaction, DMF had to be suitably dried, by stirring over calcium hydride (5.0% w/v) overnight, filtering and distilling (56^{0}C at 20mmHg) on to K-Al-silicate porous platelets.

2.3.5(ii) *The conjugation procedure*

The procedure for conjugation of tetragastrin with cholic acid was as described previously (Fig. 2-8), with the exception that 1.1 equivalents of DCC was reacted with 1.0 equivalent of cholic acid, followed by the immediate addition of 1.0 equivalent of N-hydroxysuccinimide and 1.0 equivalent of tetragastrin. A control sample was also produced by omitting the addition of tetragastrin, thus allowing a

direct comparison with the conjugation sample. The conjugation and control samples were left for an hour in order to allow the reaction to occur, before filtering to remove any DCU that may have formed. After removal of DCU, the two samples were transferred to the fridge where they were stored overnight before re-filtering. DMF was then evaporated from the two samples under vacuum, resulting in the production of a white and yellow crystalline powder (control and conjugation sample, respectively).

2.3.5(iii) *Analysis of products*

The conjugation sample was subjected to analysis by fast atom bombardment mass spectrometry (FAB-MS). Prior to analysis, the sample was dissolved in glycerol at a concentration of approximately 1mg ml^{-1}, and about 50µl of this was placed on the tip of a probe, which was subsequently inserted into the high vacuum of the mass spectrometer. In this case, the measurements were carried out on a JEOL 505 HA instrument.

The sample solution was then bombarded with a beam of fast xenon atoms. This was achieved by first ionising xenon atoms and accelerating the resulting ions through a xenon gas chamber where charge transfer occurred (equation 2-4), to give fast atoms. The excess of fast xenon ions were deflected, thereby leaving a beam of fast atoms.

$$Xe^{+\bullet}\,(fast) + Xe\,(thermal) \longrightarrow Xe\,(fast) + Xe^{+\bullet}\,(thermal) \qquad \text{(eq. 2-4)}$$

The removal of charge occurs immediately prior to the atoms striking the sample, thus they are neutral at this moment. The bombardment of fast atoms on to the metal plate coated with the substance to be investigated results in the large amount of kinetic energy in the atoms to be dissipated in various ways. For example, the vaporisation by momentum transfer and ionisation of the sample can be achieved.

Glycerol was used as a solvent, since it has been noted that the best results are obtained by coating the plate with a relatively involatile liquid matrix, and mixing the substance under investigation into the liquid matrix (Paquette, 1995). One drawback with this, however, is that a background of peaks resulting from the presence of glycerol are obtained, which must be taken into account.

The spectra of the sample obtained by FAB-MS was studied and the presence of the tetrapeptide, Trp-Met-Asp-Phe amide was indicated by the small peak at 597 m/z, and that of the ethyl ester of cholic acid by the large peak at 437 m/z (Fig. 2-24). (For ease of referral, Table 2-1 is reproduced below.) The appearance of an ion at 1176 mass units (Fig. 2-24) seemed to suggest the presence of two peptide units condensed; that is, the formation of tetrapeptide dimers (equation 2-5). Expansion of the region around the peptide dimer (Fig. 2-25) revealed the presence of a small ion at 988 mass units. This was the very close to the expected mass of the cholic acid-tetrapeptide conjugate.

Table 2-1: Molecular weights of components of conjugation sample

COMPONENT	MOLECULAR WEIGHT
Cholic acid	408
DCC	206
N-Hydroxysuccinimide	115
Tetragastrin	596
Tetragastrin-cholic acid conjugate	*986*

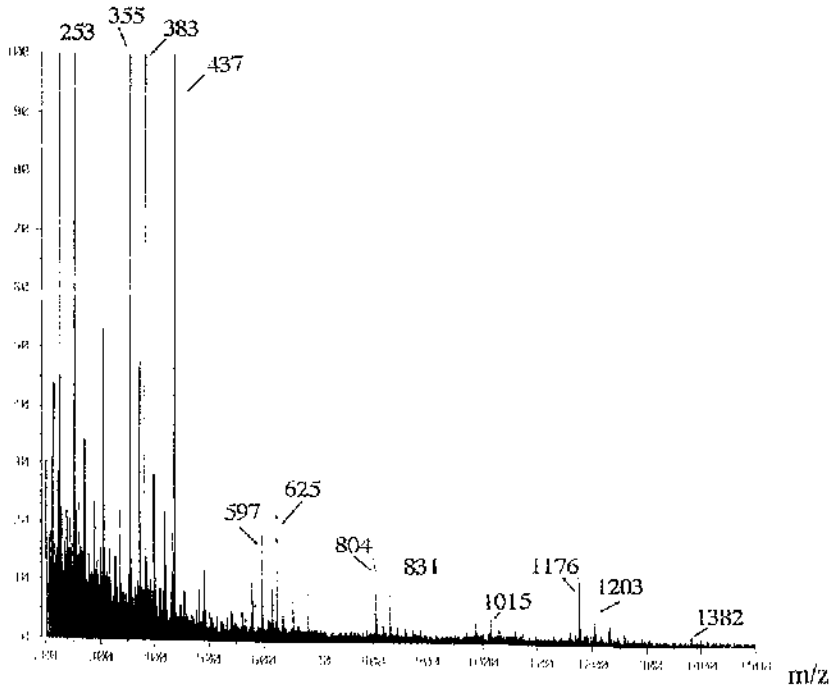
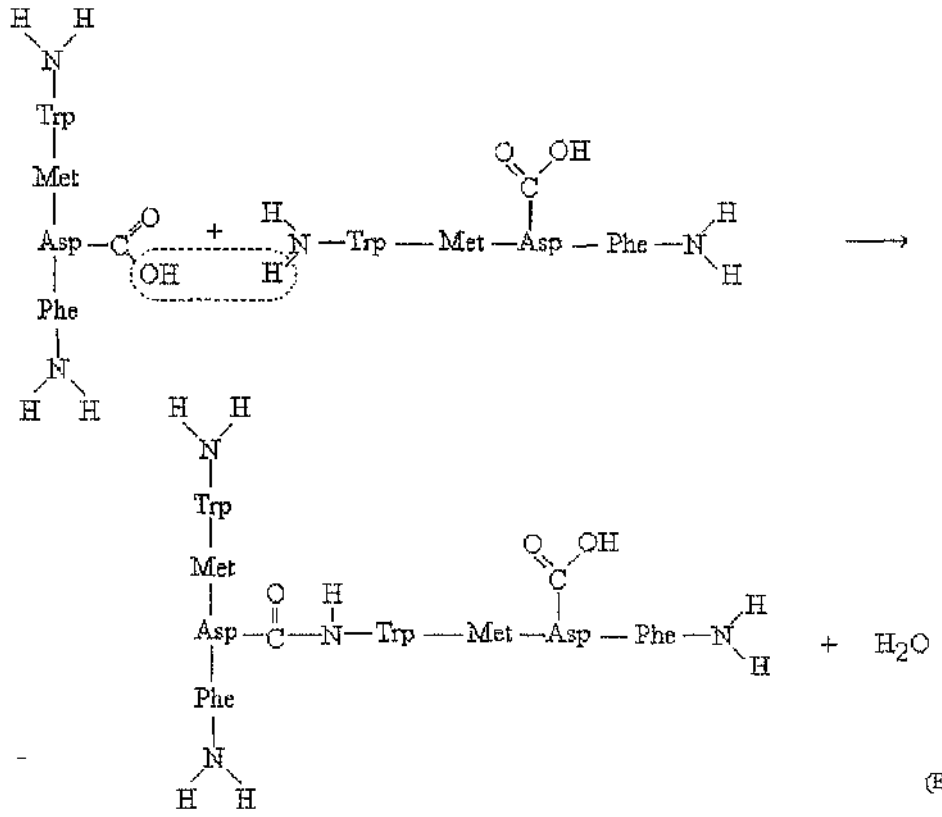


Fig. 2-24: FAB-MS spectrum of conjugate sample. Spectrum is plot of relative abundance, with the largest peak assigned 100%, against the molecular weight.



(Equation 2-5)

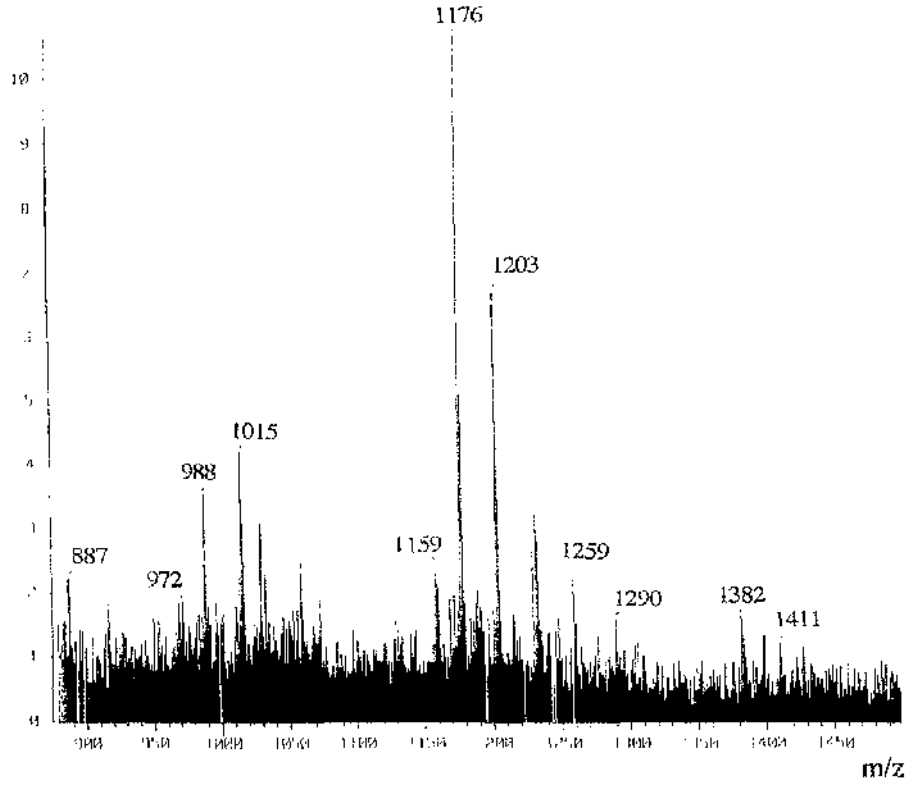


Fig. 2-25: FAB-MS spectrum of conjugation sample showing expansion of the region around the peptide dimer (1176m/z). Spectrum is plot of relative abundance, with the largest peak assigned 100%, against the molecular weight.

2.3.6 Tetragastrin-cholic acid conjugation - further alterations to the procedure

It was apparent that in order to increase the yield of the cholic acid-tetragastrin conjugate, it would be necessary to modify the procedure further. Prevention of the dimer formation may be achieved by blocking the carboxyl group (-COOH) belonging to aspartate, leaving the amino group ($-NH_2$) of tryptophan free to bind to the carboxyl group of cholic acid. However, this would be a very specialised task, preferably carried out prior to the synthesis of the tetrapeptide. Another option was, by use of HPLC, to monitor the time course of completion of the reaction occurring between DCC and cholic acid resulting in formation of the acid anhydride (see Equation 2-1), prior to addition of N-hydroxysuccinimide and tetragastrin. We could thereby assess when cholic acid would be optimally suitable for conjugation with tetragastrin. Unfortunately, this method was not feasible since the practice of HPLC required solubilization of the reagents in water; however, water would interact with the active intermediary of the peptide-cholic acid conjugation reaction, resulting in a large reduction in the yield of the peptide-cholic acid conjugate formed.

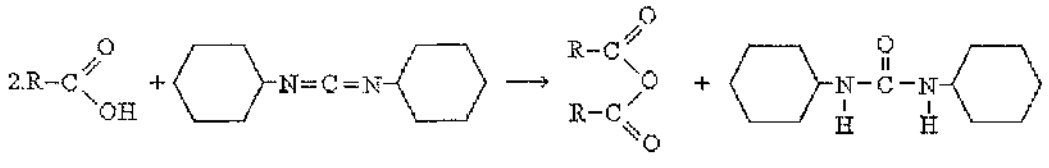
Two alternative modifications to the conjugation procedure were decided upon in an attempt to increase the yield of the required cholic acid-peptide conjugate.

2.3.6(i) *Extended reaction time of procedure*

The first of these was to extend the time interval between combining DCC (1.1 equivalent) and cholic acid (1.0 equivalent), prior to the addition of N-hydroxysuccinimide and tetragastrin (both 1.0 equivalent) to two hours rather than in immediate succession, as was the case previously. In doing this, we assumed that the reaction between DCC and cholic acid had proceeded almost to completion, if not entirely. (Had the use of HPLC been appropriate, we could have monitored the time period necessary for the completion of the first stage of the reaction.) As described previously, the final sample was refrigerated overnight before filtering to remove any white DCU precipitate. It was then transferred to a vacuum where DMF was evaporated, leaving a yellow, crystalline product. This was analysed using FAB mass spectrometry.

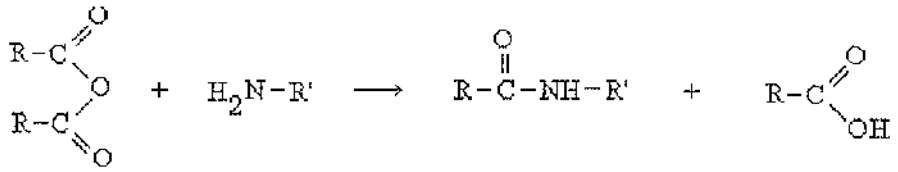
2.3.6(ii) *Omission of N-hydroxysuccinimide from procedure*

A second sample was also produced whereby N-hydroxysuccinimide was omitted in order to determine whether this could improve the yield. The conjugation reaction differs from that described in Section 2.3.2, in that the cholic acid anhydride, formed by reaction of cholic acid with DCC (Equation 2-6), reacts directly with tetragastrin to form the tetragastrin-cholic acid conjugate (Equation 2-7).



cholic acid | DCC | cholic acid anhydride | DCU

(Equation 2-6)



cholic acid anhydride | peptide | peptide-cholic acid conjugate | cholic acid

(Equation 2-7)

In order for this to be carried out with some degree of success, it was necessary to alter the molarity of the reagents involved in the reaction. With reference to equation 2-6, it was noted that 2 cholic acid molecules react with one DCC molecule to produce one molecule of cholic acid anhydride. This being the case, 2.2 equivalents of cholic acid were combined with 1.0 equivalent of DCC. (Cholic acid was slightly in excess in order to ensure that no DCC was remaining at completion of the reaction. Leftover DCC would be problematic in that it is a very harmful substance, whereas remaining cholic acid provides little difficulty.) The sample containing DCC and cholic acid was shaken for 30 minutes prior to the addition of tetragastrin (slightly in excess of 1.0 equivalent to ensure as great a yield as possible), and the final sample refrigerated overnight before filtering off solid DCU and transferring to a vacuum to

evaporate DMF. This left a yellow crystalline product, similar to that seen previously, which underwent analysis using FAB-MS.

2.3.6(iii) *Analysis of products*

Figure 2-26 (upper trace) illustrates the results of the mass spectrometry analysis of the sample in which the time period between addition of N-hydroxysuccinimide and tetragastrin to cholic acid and DCC was extended to 2 hours. There is apparently little evidence of an ion at 986m/z, which would correspond to the cholic acid-tetragastrin conjugate. This is further verified by Figure 2-26 (lower trace), which displays the results obtained over an enlarged scale. It was thereby assumed that the method had been unsuccessful.

However, Figure 2-27 demonstrates the outcome of the analysis of the sample produced by the second modified procedure in which N-hydroxysuccinimide was omitted, and provides evidence to suggest the presence of the desired conjugate, indicated by a small peak at 989m/z. The size of the yield of the conjugate was difficult to determine at this stage, since due to the technique of analysis employed, the results were qualitative rather than quantitative, in that ionic compounds were under-represented, whereas covalent compounds were fully represented. The modified procedure had proved to be more successful, with the synthesis of a quantity of the tetragastrin-cholic acid conjugate.

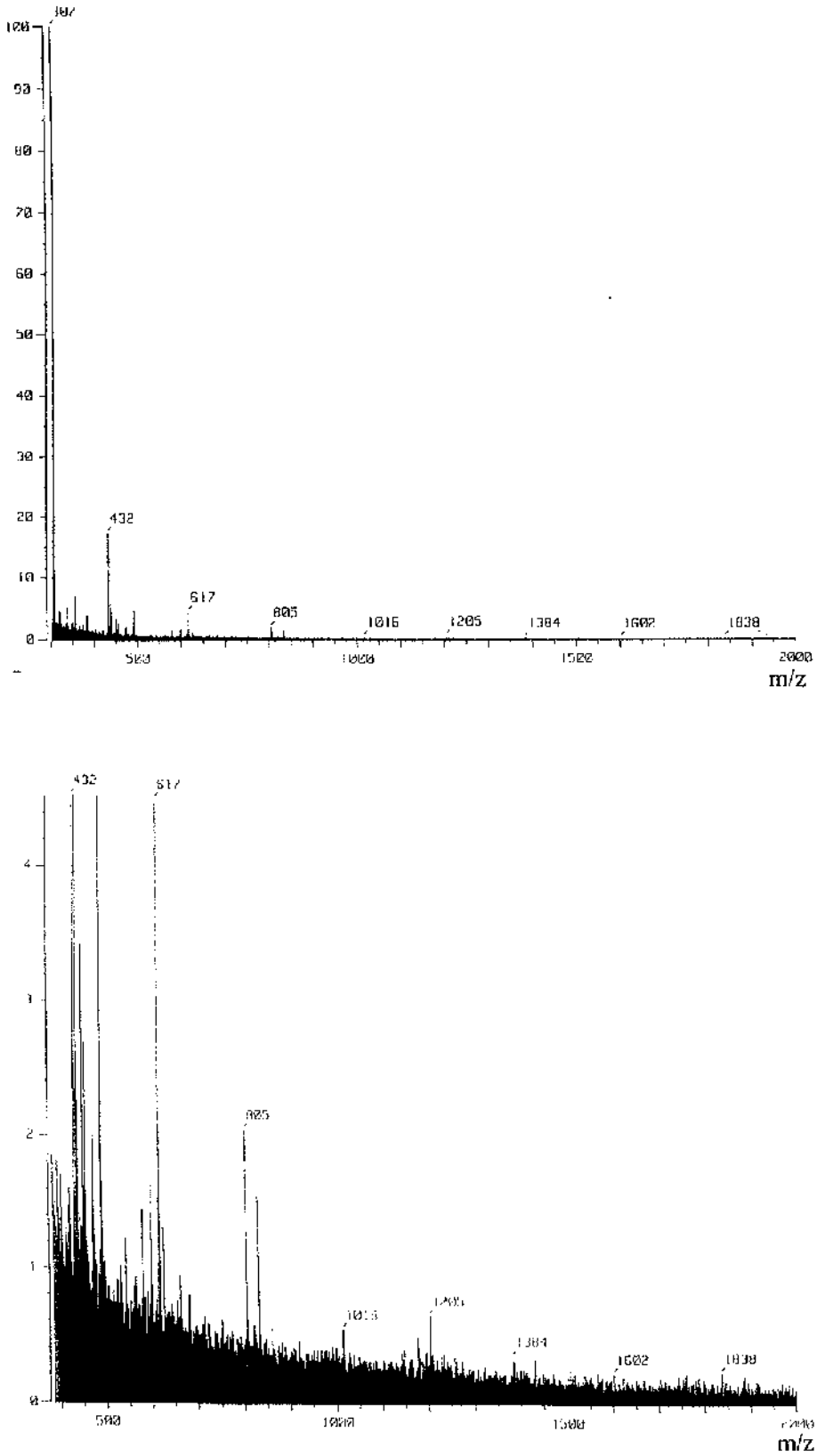


Fig. 2-26: Spectrum obtained after carrying out FAB-MS of sample in which the time period between addition of N-hydroxysuccinimide and tetragastrin to cholic acid and DCC was extended to 2 hours. (Lower trace identical to upper, but over enlarged vertical scale.) Spectrum is plot of relative abundance, with the largest peak assigned 100%, against the molecular weight.

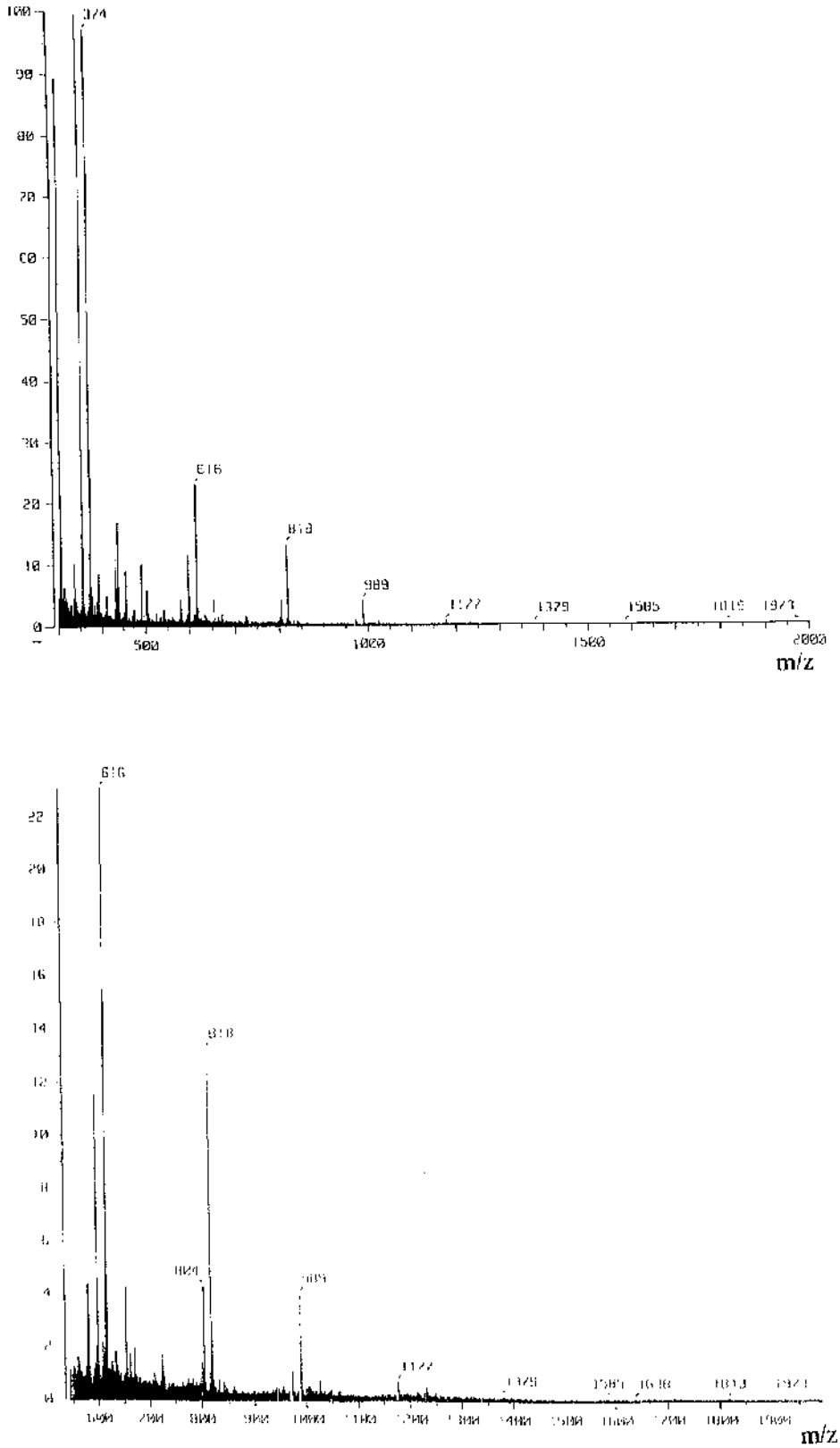


Fig. 2-27: Spectrum obtained after carrying out FAB-MS of sample in which N-hydroxysuccinimide was omitted from the reaction. (Lower trace illustrates same spectrum, but over an enlarged vertical scale.) Spectrum is plot of relative abundance, with the largest peak assigned 100%, against the molecular weight.

By contrast with HPLC for tetragastrin shown in Fig. 2-14, HPLC (as described previously) showed a considerable number of peaks (Fig. 2-28), the identity of which could be confirmed only by mass spectrometry of each peak. Thus, the complex nature of the sample suggested that the extraction and purification of the conjugate would be a time consuming, and not entirely successful process.

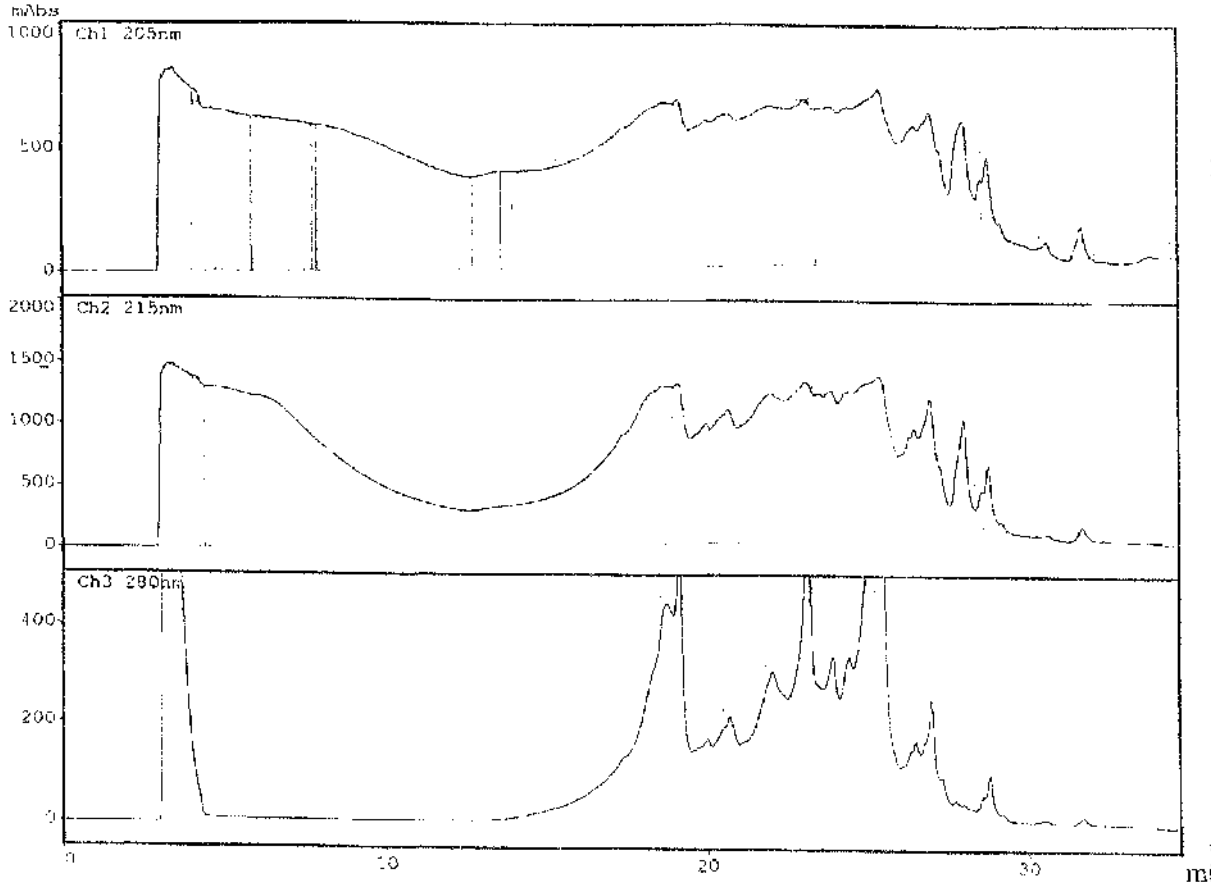


Fig. 2-28: HPLC of sample in which N-hydroxysuccinimide was omitted from reaction at a gradient of 20-40% in 0.05% TFA/H_2O and 0.05% TFA/MeCN. Detection using UV monitoring at 205nm (upper trace), 215nm (middle trace) and 280nm (lower trace). Axes indicate relative abundance against time of elution in min.

2.3.7 Solid-Phase Synthesis of Tetragastrin-Cholic acid Conjugate

We were able to gain access to a newly-acquired continuous-flow peptide synthesiser (Pioneer Peptide Synthesis Systems - PerSeptive Biosystems) which provided an alternative method for the manufacture of tetragastrin-cholic acid conjugate. This

proved to be simpler, and minimised complications such as the formation of large numbers of unknown compounds, which had dogged our previous attempts.

The synthesiser performs solid-phase synthesis, in which peptide chains are assembled, on a solid support, from the C-terminus, one amino acid at a time, elongating the chain towards the N-terminus. On completion of synthesis, the peptide is cleaved from the support to allow isolation of the product.

2.3.7(i) *Steps behind solid phase synthesis*

The α-amino group of the amino acids used in this procedure were temporarily protected by a 9-fluorenylmethoxycarbonyl (Fmoc) group, thus

$$\underset{}{\text{Fmoc}} + \underset{\text{amino acid}}{\text{H}_2\text{N-R}^1\text{-COOH}} \rightarrow \underset{\text{Fmoc-amino acid}}{\text{Fmoc-NH-R}^1\text{-COOH}}$$

In addition, the form of aspartate used was that in which the carboxyl side chain was prevented from participation in the reaction by esterification with tertiary butanol, thus

$$\underset{\text{Fmoc-aspartate}}{\text{Fmoc-NH-R(COOH)-COOH}} + \underset{\text{tertiary butanol}}{\text{HO-C(CH}_3)_3} \longrightarrow \underset{\text{side chain protected Fmoc-aspartate}}{\text{Fmoc-NH-R(C(=O)-O-C(CH}_3)_3\text{)-COOH}} + \text{H}_2\text{O}$$

In the first cycle, attachment of the C-terminal amino acid, Fmoc-phenylalanine, to the solid support occurred by way of a linker molecule. In the present study, a PAL linker[2] [5-(4-Fmoc-aminomethyl-3, 5-dimethoxy-phenoxy) valeric acid] was used, the structure of which is shown in Fig. 2-29.

[2] *The PAL linker yields deprotected C-terminal amide peptides. These peptides have structural components common to both those peptides yielded by the xanthenyl amide linker (XAL), and to those yielded by the peptide acid support linker (PAC).*

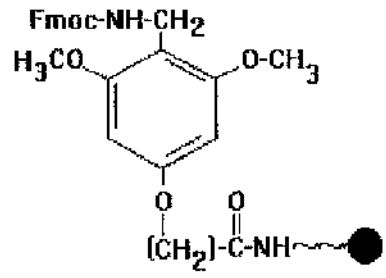


Fig. 2-29: PAL linker structure (~~• denotes polystyrene support).

The free end of the PAL linker has an amine group protected with an Fmoc group. During the first cycle of synthesis, the Fmoc group of the PAL linker was removed using the secondary amine, piperidine, to expose a free amine group. The carboxyl group of Fmoc-phenylalanine was activated and coupled by a condensation reaction with the amine group of the PAL linker using *O*-benzotriazol-1-yl-N, N, N', N'-tetramethyluronium tetrafluoroborate (TBTU), in the presence of 1-hydroxybenzotriazole (HOBt), as the coupling agent.

The Fmoc group attached to the N-terminus of phenylalanine was removed using piperidine, and the carboxyl group of Fmoc-aspartate was activated and coupled with the exposed amine group of phenylalanine (the side chain protecting group was left intact), again using TBTU in the presence of HOBt.

This cycle was repeated (as shown in Fig. 2-30) until the required number of amino acids had been added which, in the present case was four (tryptophan-methionine-aspartate-phenylalanine). Within each cycle, the reactor was, at the appropriate time, flushed through to remove excess amino acids and Fmoc. Fuller details are provided in the reference manual (Pioneer™ Peptide Synthesis System User's Guide).

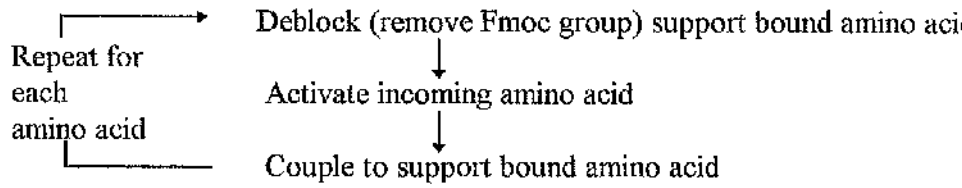
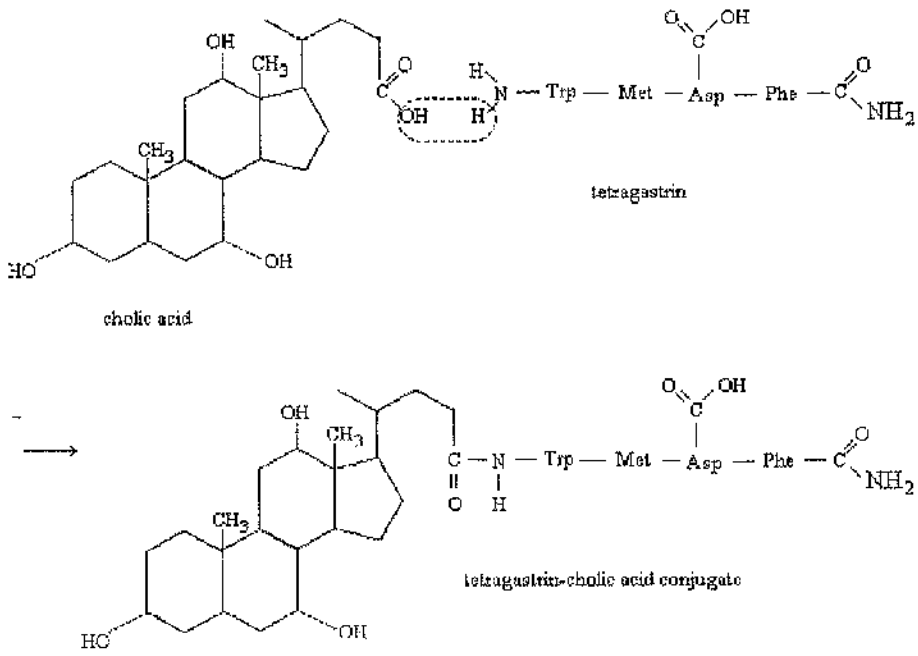


Fig. 2-30: Diagram to summarise the manufacture of tetragastrin using the continuous-flow peptide synthesiser.

The final stage of the reaction was the attachment of cholic acid to the N-terminus of tryptophan. The Fmoc group of tryptophan was removed using piperidine, and coupling of the terminal carboxyl group of cholic acid with the amine group of tryptophan occurred using TBTU in the presence of HOBt. The condensation reaction involved is shown by Equation 2-8.



(Equation 2-8)

Following completion of the tetragastrin-cholic acid conjugate, it was necessary to cleave the compound from the PAL linker and polystyrene support, and remove the tertiary butanol from the side chain of aspartate. This was done under extremely acidic conditions, using trifluoroacetic acid (TFA) (pKa of 0.5), as quickly as possible in order to minimise the exposure of the peptide to the acidic reagent.

A 40mg sample was produced in this way. A ninhydrin test on the polystyrene support of the synthesiser, on completion of synthesis, was carried out and found to be negative, thereby demonstrating that all free amino acids had been utilised in the synthesis. The composition of the sample was analysed using FAB mass spectrometry.

2.3.7(ii) *Analysis of sample using FAB mass spectrometry*

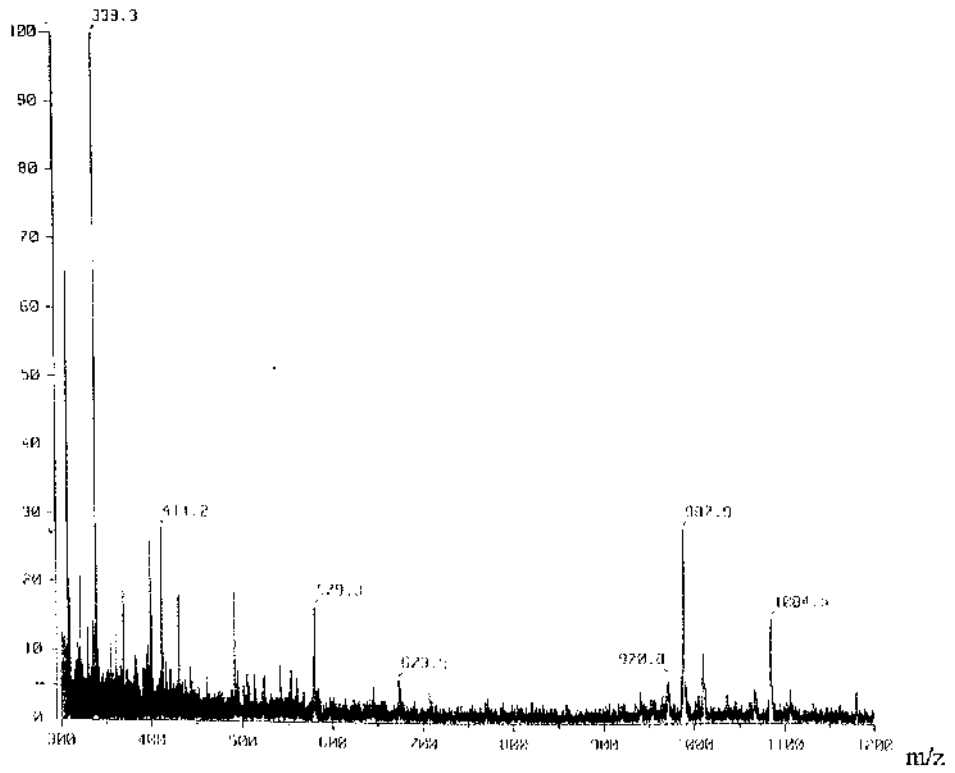


Fig. 2-31: Spectrum of sample, synthesised using continuous-flow peptide synthesiser, as obtained by FAB-MS. Spectrum is plot of relative abundance, with the largest peak assigned 100%, against the molecular weight.

Figure 2-31 illustrates the results obtained from such an analysis. The spectrum obtained appeared to be very much cleaner than the spectra for previous attempts (Figs. 2-24 to 2-27), although several peaks relating to different ions were found. However, most importantly, an ion at 987m/z was evident, and attributed to the cholic acid-tetragastrin conjugate: this was evidence indeed that the conjugation procedure had been successful (for ease of referral, Table 2-1 is reproduced below).

Table 2-1: Molecular weights of components of conjugation sample

COMPONENT	MOLECULAR WEIGHT
Cholic acid	408
DCC	206
N-Hydroxysuccinimide	115
Tetragastrin	596
Tetragastrin-cholic acid conjugate	*986*

The other peaks presented, notably at approximately 338, 411, 579 and 673m/z were more difficult to define; however, the process of FAB mass spectrometry may result in the fragmentation of the compound under analysis, and it was thought that this may account for these peaks. For example, fracture of the conjugate at the bond linking tryptophan and methionine, resulting in the production of a cholic acid-tryptophan compound, and a separate tripeptide, might account for the ions at 411 and 579m/z. Expansion of the spectrum scale (Fig. 2-32) revealed larger ions at approximately 1010, 1067, 1084, 1106 and 1180m/z. Again, these were difficult to characterise, although the ion at 1180m/z was likely to be the tetragastrin dimer, previously described. The ion at 1084m/z may be due to trifluoroacetylation of the conjugate, since this would increase the mass by 97m/z. (This would have arisen during the removal of the tertiary butyl protecting group from the amino acid side chains, and also when detaching the final sample from the polystyrene support.) To reiterate, we were unable to determine the actual yield of each of these components since, due to the technique of analysis employed, ionic compounds were under-represented, whereas covalent compounds were fully represented.

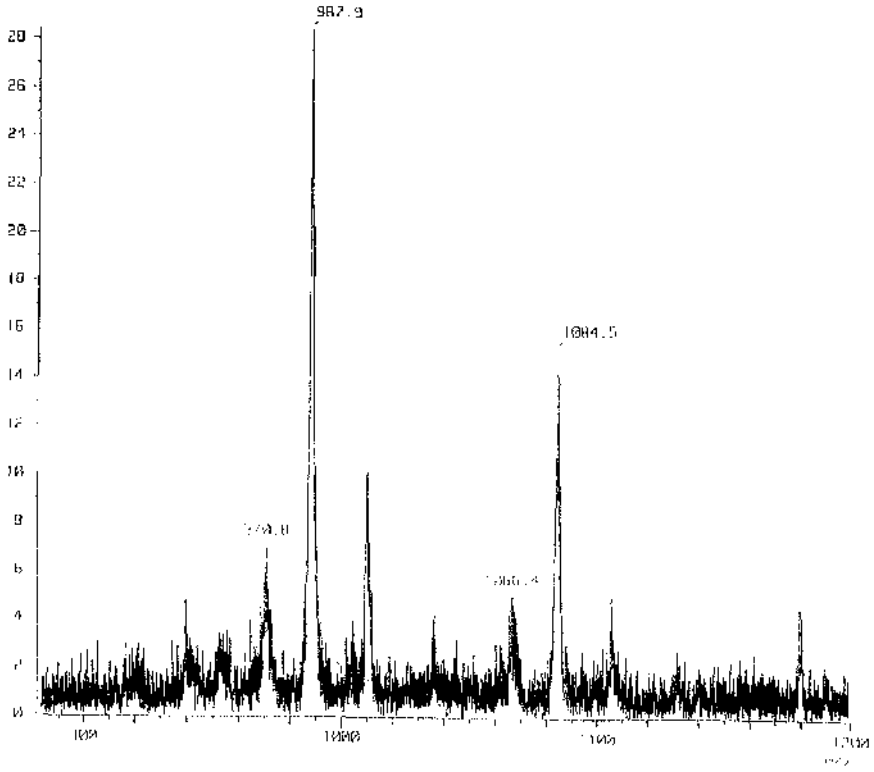


Fig. 2-32: Spectrum of sample, synthesised using continuous-flow peptide synthesiser, over an enlarged horizontal and vertical axis, as obtained by FAB-MS.

2.3.7(iii) *Analysis of sample using HPLC system*

We required assurance that our sample, synthesised in the continuous-flow peptide synthesiser, was reasonably pure, given that many of the ions disclosed in the FAB mass spectra presumably occurred as a consequence of fragmentation. It was decided, therefore, to carry out further analysis using HPLC, as described previously. Figure 2-33 demonstrates the chromatograms obtained at wavelengths 205, 215 and 280nm. As has been stated, HPLC analysis at 205nm is used for detection of compounds containing peptide bonds, 215nm is the traditionally selected option, and 280nm discloses compounds containing aromatic groups, such as phenylalanine and tryptophan. Thus, the chromatogram at 280nm provides the best guide for assessment of our sample.

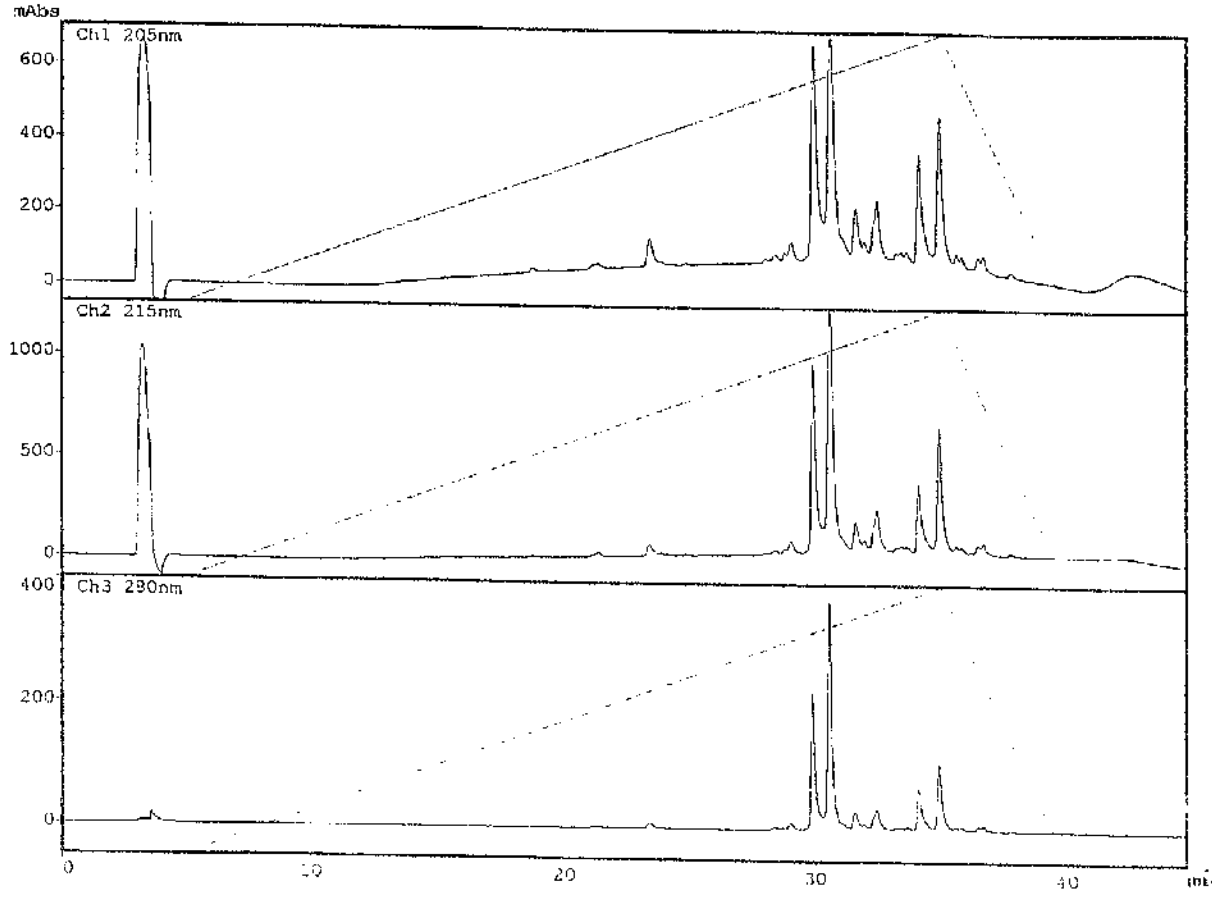


Fig. 2-33: HPLC of the sample, synthesised using continuous-flow peptide synthesiser, at a gradient of 20-40% in 0.05% TFA/H_2O and 0.05% TFA/MeCN. Axes indicate relative abundance against time of elution in min.

It was clear that there is not a single species present, but rather three pairs of peaks. It may be suggested that the pairs of peaks arose as the result of the formation of two species of tetragastrin of the same molecular weight, as shown by the HPLC of the Sigma tetragastrin sample (Fig. 2-14), which was presumably manufactured in a similar way to the present sample. The multiple pairs of peaks indicate, in addition, two errors of synthesis of the tetragastrin-cholic acid conjugate, the identity of which could be made only by collecting HPLC fractions and performing mass spectrometry on them. However, on an intuitive basis, inspection of the trace detected at 280nm (Fig. 2-33), would suggest that the major pair of peaks at 30 min represents the tetragastrin-cholic acid conjugate, while the other two pairs of peaks represent errors of synthesis.

Given the normal high efficiency of this method of chemical synthesis, namely, that it manufactures peptides of very high purity, we were constrained not to be deflected into seeking the identity of the different peaks observed with HPLC. We accepted, for the purpose of this study, that the sample produced by continuous-flow peptide synthesis was sufficiently pure.

RESULTS

3.1 Physiological experiments

3.1.1 Gastric acid secretion in response to tetragastrin

Initially, we required to determine the increase in gastric acid secretion above basal levels in response to intravenous injection of the test peptide, tetragastrin (Trp-Met-Asp-Phe amide), in the anaesthetised rat. The minimum effective dose of tetragastrin was established by first administering intravenously a bolus injection of 12.5μg kg^{-1} tetragastrin, and observing the effect on gastric acid secretion, which was measured for consecutive 15 min periods. If this was ineffective in initiating an unequivocal response, the dose was increased stepwise to a maximum of 60μg kg^{-1}. A second I.V. injection of the same dosage, prior to completion of the experiment, was used to confirm the viability of the preparation.

3.1.1(i) *The effect of tetragastrin (I.V.) on gastric acid secretion*

A typical experiment (Figure 3-1) demonstrated that injection of tetragastrin (20μg kg^{-1}, I.V.) (first arrow) caused an increase in gastric acid secretion to a peak value of 0.73μmol $15min^{-1}$ above baseline, and resulted in a cumulative increase in acid secretion of 2.57μmol hr^{-1}. The response remained elevated above control values for 60 minutes. In order to test for reproducibility of the response, a second

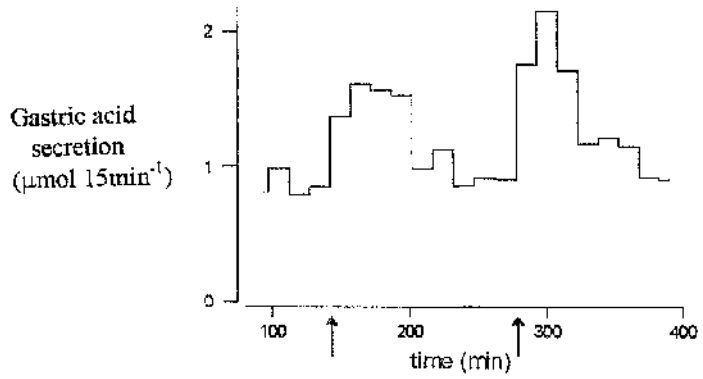


Fig.3-1: The effect on intravenous tetragastrin (20μg kg^{-1}, first and second arrows) on gastric acid secretion in the anaesthetised rat (rat 5 in Table 3-1).

identical injection of tetragastrin was administered (second arrow) 135 minutes after the first. This was seen to produce a peak increase in acidity of 1.26μmol $15min^{-1}$, 1.7 times larger than the peak response seen in response to the initial injection of tetragastrin. The peak increase in acidity in response to both the first and second injection was observed to occur in the second collection period, that is between 15 and 30 minutes after introduction of the tetrapeptide into the venous system. However, the response to the second injection of tetragastrin remained elevated above baseline for a duration of 90 minutes, and a cumulative gastric acid secretion totalling 3.24μmol hr^{-1} was seen, demonstrating a 1.3-fold increase over the previous cumulative response.

Table 3-1: Peak and cumulative (cum.) increases in gastric acid secretion in response to intravenous tetragastrin (G4) (minimum effective dose) as a first and second procedure.

Rat	Initial I.V. G4		Second I.V. G4	
	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})
1	0.13	0.46	0.76	1.52
2	0.62	1.57	1.12	3.21
3	0.46	0.69	0.87	1.3
4	0.72	1.62	0.49	1.97
5	0.73	2.57	1.26	3.24
Mean ± S.D.	0.53 ± 0.25 (P=0.009)	1.38 ± 0.84 (P=0.021)	0.90 ± 0.30 (P=0.003)	2.25 ± 0.92 (P=0.006)

P-values denote statistical significance for comparison with baseline.

Similar experiments were carried out on a total of 5 animals, the results of which are presented in Table 3-1. A further animal died prior to completion of the experiment. The mean values demonstrated that an initial injection of tetragastrin (minimum effective dose, I.V.) resulted in a significant peak increase in gastric acid levels, above baseline, to 0.53 ± 0.25μmol $15min^{-1}$ (P=0.009). A mean cumulative response of 1.38 ± 0.84μmol hr^{-1} was found to be significant (P=0.021). A subsequent injection of tetragastrin (the same min. effective dose, I.V.) was administered between 65 and 180 minutes later (actual period of injection was

dependent on the time taken for the baseline to settle after the response to the first injection), and led to an increase in peak gastric acid levels of 0.90 ± 0.30μmol $15min^{-1}$ (P=0.003). Increased gastric acid levels over a period of one hour following administration were found to total a mean cumulative value of 2.25 ± 0.92μmol hr^{-1} (P=0.006). A paired t-test demonstrated that the peak increases in gastric acid secretion in response to the first and second intravenous injection were not statistically different (P=0.075), whereas the cumulative responses to the second intravenous injection were considered to be significantly larger than the response to the first (P=0.018). This was likely to be due to the fact that the duration of the increase in acid secretion in response to the first injection of tetragastrin was typically of shorter duration than that in response to the second (a mean value of 51 minutes compared with 81 minutes).

3.1.1(ii) *Effect of intra-ileal (I.I.) infusion of tetragastrin on gastric acid secretion*

It was central to the project to determine whether biologically active tetragastrin could be absorbed across the wall of the small intestine, thereby enhancing gastric acid secretion. In order to investigate this, a series of experiments was undertaken in which the following protocol was followed. First, the minimum effective dose of tetragastrin (between 12.5 and 60μg kg^{-1}) was administered intravenously, and gastric acid levels were shown to increase, as was anticipated. On return to baseline, 600μg kg^{-1} tetragastrin (in 1.0ml isotonic saline) was infused into the ileum via a cannula inserted close to the ileo-caecal junction (see Methods). After an interval of 90-105 minutes, a subsequent injection of tetragastrin (same minimum effective dose, I.V.) was administered.

Figure 3-2 illustrates a typical response to such a procedure. The initial dose of tetragastrin (15μg kg^{-1}, I.V.) (first arrow) resulted in an elevated gastric acid level to a peak value of 1.11μmol $15min^{-1}$, occurring in the second collection period. The overall response lasted for a duration of 45 minutes, giving a cumulative rise in total acidity above baseline of 2.02μmol hr^{-1}. Following the return of gastric acid secretion to pre-injection levels, administration of ileally-infused tetragastrin

(600μg kg^{-1} in 1.0ml isotonic saline) (second arrow) had no discernible effect on gastric acid secretion which actually fell to slightly below basal level by 0.01μmol over the 90 minute duration, prior to subsequent intravenous infusion of tetragastrin (15μg kg^{-1}) (third arrow). This second intravenous injection resulted in a peak increase in total acidity of 1.14μmol $15min^{-1}$, which occurred in the collection period in which the injection was given. The increase in gastric acid secretion lasted for 45 minutes before falling back to the control level. A cumulative response of 2.43μmol hr^{-1} was thereby attained.

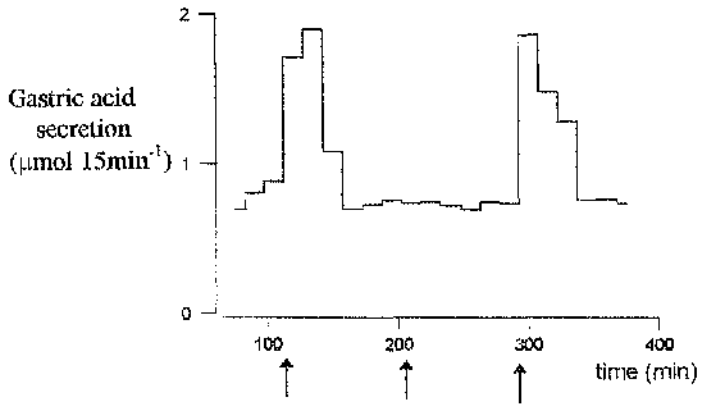


Fig. 3-2: The effect of intravenous tetragastrin (15μg kg^{-1}, first and third arrows), and ileally-infused tetragastrin (600μg kg^{-1}, second arrow) on gastric acid secretion in the anaesthetised rat (rat 4 in Table 3-2).

Eleven such experiments were carried out following the above standard procedure (the experiment did not go to completion in a further two rats); the results are presented in Table 3-2. Overall, the initial intravenous injection of tetragastrin (15, 30 or 60μg kg^{-1}) resulted in a significant increase of gastric acid levels above baseline to a mean peak value of 0.58 ± 0.33μmol $15min^{-1}$ (P=0.001), and a mean cumulative acidity of 1.27 ± 0.83μmol hr^{-1} (P=0.001). With the exception of three animals, in which the peak increase was present in the second collection period (i.e. the collection period following the one in which the injection was administered), the peak increase in acidity was found to occur in the period of injection. The mean duration of the response was 48 minutes, a value comparable to that of the response to the initial intravenous injection of tetragastrin in the previous experiment. In response to ileal infusion of tetragastrin (600μg kg^{-1}), a mean drop

in total acidity of 0.26 ± 0.40μmol 90min^{-1} was found not to be significant (P=0.055). Subsequent infusion of tetragastrin (15, 30 or 60μg kg^{-1}, I.V.) resulted in a significant increase in gastric acid levels of 0.81 ± 0.53μmol 15min^{-1} (P=0.001). This occurred typically in the period of injection, although one rat presented a peak response in the fourth collection period, 45 to 60 minutes after injection. However, a sizeable response was present in the first collection period of 0.48μmol 15min^{-1}. The duration of response was difficult to determine, since in only four rats did the gastric acid levels return to pre-injection levels whereas, in the other seven, acid levels remained elevated. However, a significant cumulative increase in total acidity of 2.43 ± 1.86μmol hr^{-1} (P=0.002) resulted.

Table 3-2: Peak and cumulative (cum.) increases in gastric acid secretion in response to ileally-administered tetragastrin (G4) (600μg kg^{-1} in 1.0ml saline) over 90 minute period, and to intravenous tetragastrin (15, 30 or 60μg kg^{-1}) as a first and final procedure.

Rat	Initial I.V. G4		Ileal G4	Second I.V. G4	
	Peak increase (μmol 15min^{-1})	Cum. increase (μmol hr^{-1})	Cumulative increase (μmol 90min^{-1})	Peak increase (μmol 15min^{-1})	Cum. increase (μmol hr^{-1})
1	0.44	0.87	-0.28	0.52	1.59*
2	0.85	2.82	-0.31	2.01	6.95*
3	1.01	2.37	-0.68	0.93	3.11*
4	1.11	2.02	-0.01	1.14	2.43
5	0.31	0.49	0.01	0.20	0.47
6	0.49	1.21	-1.25	0.50	1.18
7	0.33	0.89	0.00	0.30	0.93*
8	0.27	0.48	-0.43	0.40	1.30*
9	0.28	0.31	-0.06	0.56	1.54
10	0.30	0.88	0.09	1.17	3.99*
11	0.96	1.63	0.01	1.13	3.25*
Mean ± S.D.	0.58±0.33 (P=0.001)	1.27±0.83 (P=0.001)	-0.26 ± 0.40 (P=0.055)	0.81±0.53 (P=0.001)	2.43±1.86 (P=0.002)

P-values denote statistical significance for comparison with baseline.

* *denotes establishment of new baseline which was elevated compared with that at start of experiment.*

A paired t-test demonstrated that, although the mean peak responses to the first and second intravenous injections were not significantly different from each other, the cumulative responses were (P=0.095 and P=0.018, respectively). As before,

the elevation of acid levels over a longer period of time in response to the second intravenous injection would appear to account for this.

From this series of experiments, we deduced that ileally-infused tetragastrin was unable to cross the wall of the ileum over a 90 minute sampling period, and therefore showed no discernible effect on gastric acid levels. However, the significantly greater mean cumulative increase in gastric acid levels in response to the second intravenous injection of tetragastrin indicates the possibility that potentiation of the response may have occurred by the absorption of biologically active tetragastrin across the ileal wall. It may be the case that a duration of 90 minutes, in which the effects of ileally-infused tetragastrin were measured, was not sufficient to allow the passage of tetragastrin, or perhaps subliminal levels permeated the ileal wall but were ineffective in inducing a perceivable enhancement of gastric acid secretion. Experiments were subsequently carried out to investigate these questions.

3.1.1(iii) *Effect of a higher dose of tetragastrin (I.I.) on gastric acid secretion*

A series of experiments similar to the aforementioned protocol was undertaken on four rats. As before, the minimum effective dose (in this case, 12.5μg kg^{-1}) was administered intravenously; however, 2500μg kg^{-1} tetragastrin (in 5.0ml) was then infused into the ileum and gastric acid secretion was followed over a period of 90 minutes, in order to determine whether the increased total amount of tetragastrin would raise gastric acid levels above baseline values. This was followed by a second intravenous injection of tetragastrin (12.5μg kg^{-1}), as described previously.

An example of the outcome of such an experiment is shown in Figure 3-3. Intravenous administration of tetragastrin (12.5μg kg^{-1}) (first arrow) resulted in enhancement of gastric acid secretion to a peak value of 0.40μmol $15min^{-1}$ above baseline in the period of injection. The response lasted for 30 minutes before settling at a total gastric acidity elevated above pre-injection value. This amounted to a cumulative total acidity of 1.10μmol hr^{-1}. Following the re-establishment of a settled baseline, albeit at a raised value from the previous level, ileal infusion of tetragastrin (2500μg kg^{-1} in 5.0ml isotonic saline) was carried out (second arrow),

and samples collected at 15 minute intervals for a further 90 minutes. Ileal tetragastrin had no apparent effect on gastric acid levels over this period of time; a cumulative drop in acidity of 0.08μmol hr^{-1} was determined. Following this 90 minutes period, an intravenous injection of tetragastrin, identical to the first, was administered (third arrow), resulting in a peak increase in acidity of 0.39μmol $15min^{-1}$. This occurred in the period of injection, with acid levels remaining above baseline for 30 minutes before returning to pre-injection level. A cumulative increase in total acidity of 0.65μmol hr^{-1} above baseline was observed in response to this injection.

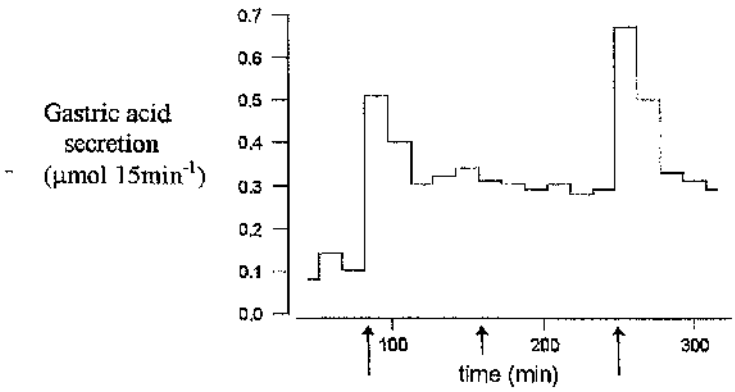


Fig. 3-3: The effect of intravenous tetragastrin (12.5μg kg^{-1}, first and third arrows), and ileally-infused tetragastrin (2500μg kg^{-1} in 5.0ml saline), second arrow) on gastric secretion in the anaesthetised rat (rat 4 in Table 3-3).

In this series of experiments, the peak response to both the first and second intravenous injection of tetragastrin (12.5μg kg^{-1}) typically occurred in the period of injection, and the increased response above baseline lasted between 30 and 45 minutes. The mean peak increase in gastric acid secretion in response to the initial procedure was 0.47 ± 0.16μmol $15min^{-1}$, and that to the second intravenous injection was 0.36 ± 0.06μmol $15min^{-1}$, both of which were significantly increased with respect to baseline (P=0.010 and P=0.002, respectively) (Table 3-3). Further, the cumulative increases in acid secretion above baseline over the hour following both the first and second intravenous injection of tetragastrin were significant when compared to baseline (1.15 ± 0.36μmol hr^{-1}, P=0.008; 0.64 ± 0.29μmol hr^{-1}, P=0.022, respectively). Similar to the previous experiments, ileal infusion of

2500μg kg^{-1} tetragastrin did not significantly enhance gastric acid secretion over a 90 minute period. Indeed, a mean cumulative drop of 0.13 ± 0.19μmol hr^{-1} was demonstrated, though this was not significant (P=0.26).

Table 3-3: Peak and cumulative (cum.) increases in gastric acid secretion in response to ileally-administered tetragastrin (G4) (2500μg kg^{-1} in 5.0ml saline) over 90 minute period, and to intravenous tetragastrin (minimum effective dose) as a first and final procedure.

Rat	Initial I.V. G4		IlealG4	Second I.V. G4	
	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})	Cumulative increase (μmol $90min^{-1}$)	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})
1	0.60	1.35	0.07	0.41	1.04*
2	0.28	0.66	-0.38	0.35	0.46
3	0.59	1.48	-0.13	0.27	0.39
4	0.40	1.10*	-0.08	0.39	0.65
Mean ± S.D.	0.47±0.16 (P=0.010)	1.15±0.36 (P=0.008)	-0.13 ± 0.19 (P=0.26)	0.36±0.06 (P=0.002)	0.64±0.29 (P=0.022)

P-values denote statistical significance for comparison with baseline.

* *denotes establishment of new baseline which was elevated compared with that at start of experiment.*

A paired t-test demonstrated that there were no significant differences either in the peak or in the cumulative responses to the first and second intravenously infused tetragastrin (P=0.29 and P=0.082, respectively). This thereby demonstrated some discrepancy when comparing the cumulative responses of this series of experiments with the cumulative responses of previous experiments. This discrepancy appears to lie with the fact that the duration of the response to the second intravenous injection, in this set of experiments, lasted for a shorter duration than in previous experiments. However, we can still have confidence that the preparation was viable in the latter stages of the experiment. Furthermore, this series of experiments provides evidence to suggest that ileal tetragastrin was unable to cross the wall of the ileum and further enhance the secretion of gastric acid in response to the second intravenous infusion.

3.1.1(iv) *Higher dose of intra-ileal tetragastrin as higher concentration over extended time period*

As before, a minimum effective dose (12.5 or 25μg kg^{-1}) of tetragastrin (I.V.) was followed by intra-ileal infusion of tetragastrin and then, finally, by a second intravenous infusion of tetragastrin. However, this time the increased dose of ileally-infused tetragastrin (2500μg kg^{-1}), now in 1.0ml isotonic saline, representing a five-fold increase in concentration, was allowed to remain in the intestine for 180 minutes, that is, almost double the length of time in previous experiments, before the second intravenous injection of tetragastrin was administered. In addition to allowing us to test further the two questions as to whether intra-ileal tetragastrin in much higher concentration increased gastric acid levels, *per se*, and whether it potentiated the response of the second intravenous injection of tetragastrin, by increasing the duration of the experiment, this would also resolve whether ileal tetragastrin absorption had, in fact, been limited by the 90 to 105 minute period allowed in the previous experiments. In order to minimise the volume of fluid injected into the small intestine, the tetragastrin was freeze-dried and then dissolved in 1.0ml isotonic saline, rather than injecting 5.0ml solution, as described previously.

Figure 3-4 demonstrates the outcome of a typical experiment. Tetragastrin (12.5μg kg^{-1}, I.V.) (first arrow) caused gastric acid secretion to increase to a peak value of 0.40μmol 15min^{-1} above baseline and, although this occurred in the third collection period following injection, a noticeable increase was observed in the collection periods prior to this (0.12μmol 15min^{-1} in the period of injection, and 0.34μmol 15min^{-1} in the second period). The response remained elevated for a total duration of 60 minutes, whereby a cumulative gastric acid secretion of 1.06μmol hr^{-1} above baseline was attained. Consistency with previous experiments resulted as regards the invariance of the level of gastric acid secretion in response to ileally-infused tetragastrin (2500μg kg^{-1} in 1.0ml isotonic saline) (second arrow).

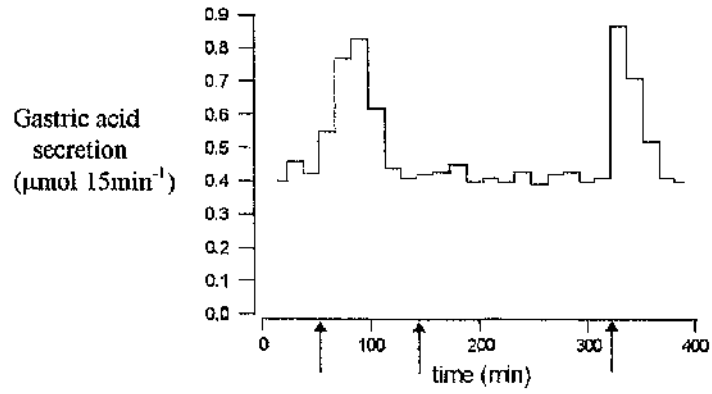


Fig.3-4: The effect of intravenous tetragastrin (12.5μg kg^{-1}, first and third arrows), and ileally-infused tetragastrin (2500μg kg^{-1} in 1.0ml saline, second arrow) on gastric acid secretion in the anaesthetised rat (rat 2 in Table 3-4).

This was ineffective in producing any noticeable change in the gastric acid secretion from basal levels over a 180 minute period. A second intravenous injection of tetragastrin, identical to the first, was administered subsequently (third arrow), and resulted in an increase in peak total acidity of 0.45μmol 15min^{-1} in the period of injection, resulting in a cumulative rise in titratable acidity above baseline of 0.85μmol hr^{-1}. Thus, the viability of the preparation at this late stage in the procedure was assured.

Table 3-4: Peak and cumulative (cum.) increases in gastric acid secretion in response to ileally-administered tetragastrin (G4) (2500μg kg^{-1} in 1.0ml saline) over 180 minute period, and to intravenous tetragastrin (minimum effective dose) as a first and final procedure.

Rat	Initial I.V. G4		IlealG4	Second I.V. G4	
	Peak increase (μmol 15min^{-1})	Cum. increase (μmol hr^{-1})	Cumulative increase (μmol 180min^{-1})	Peak increase (μmol 15min^{-1})	Cum. increase (μmol hr^{-1})
1	0.54	0.87	-0.33	0.73	1.98
2	0.40	1.06	0.00	0.45	0.85
3	0.45	1.06	-0.37	0.57	1.53
4	0.29	1.00	0.05	0.38	1.25
5	0.34	0.62	-0.46	0.53	0.79
6	0.51	0.41	-0.26	0.35	0.78
Mean ± S.D.	0.42±0.10 (P=0.001)	0.84±0.27 (P=0.001)	-0.23 ± 0.21 (P=0.043)	0.50±0.14 (P=0.001)	1.20±0.49 (P=0.002)

P-values denote statistical significance for comparison with baseline.

A total of six such experiments were carried out, and the results are tabulated in Table 3-4. A significant increase in mean gastric acid secretion, with respect to baseline, of 0.42 ± 0.10μmol $15min^{-1}$ (P=0.001) was observed in response to an initial administration of intravenous tetragastrin (12.5 or 25μg kg^{-1}). The peak response occurred in either the period of injection or the subsequent collection period (between 0 and 15 minutes, or between 15 and 30 minutes, respectively), with the exception of one rat, in which the peak response occurred in the third collection period (see Fig. 3-4). Gastric acid levels remained elevated above baseline for a duration of between 30 and 60 minutes, and a mean cumulative increase in gastric acidity was calculated as being 0.84 ± 0.27μmol hr^{-1} (P=0.001). Ileal infusion of tetragastrin (2500μg kg^{-1} in 1.0ml isotonic saline) resulted in a fall in gastric acid levels of 0.23 ± 0.21μmol hr^{-1} which was significant (P=0.043), and we can thus infer that tetragastrin was unable to cross the wall of the ileum and enter the bloodstream to any detectable extent over a 180 minute period. A second intravenous injection of tetragastrin (12.5 or 25μg kg^{-1}) resulted in a significant mean peak increase in acidity of 0.50 ± 0.14μmol $15min^{-1}$ (P=0.001), which typically occurred in the period of injection. Gastric acid secretion remained elevated above basal levels for a duration of between 45 and 60 minutes, resulting in a mean cumulative titratable acidity of 1.20 ± 0.49μmol hr^{-1} (P=0.002). By comparison of the mean peak response to the initial dose of intravenous tetragastrin with that to the second, it was determined that the mean peak response to the second I.V. injection was significantly larger (P=0.027), while the mean cumulative response was not significantly different (P=0.099).

It is therefore likely that ileal tetragastrin did not exert a detectable increase on gastric acid secretion during its presence in the ileum over 180 minutes but rather may have caused a decrease. Likewise, it did not cause an unequivocal potentiation of the increased cumulative gastric acid levels observed in response to the final intravenous injection of tetragastrin. This is shown by the ratio of the second to the first cumulative acid secretion which, in the present experiment was 1.43. This is actually less than the ratio for either the 600μg kg^{-1} intra-ileal infusion experiment (Table 3-2) which was 1.91 or the control experiment (Table

3-1) which was 1.63. Furthermore, in the present series of experiments, the sensitivity of the animal to the final intravenous injection of tetragastrin confirmed the viability of the preparation in every case.

3.1.2 Gastric acid secretion in response to intravenously administered tetragastrin-cholic acid conjugate (G-CA)

Our previous experiments have demonstrated an increase in gastric acid secretion in response to intravenous administration of between 12.5 and 60μg kg^{-1} tetragastrin as a minimum effective dose. In order to provide a direct comparison of G-CA-elicited response with the previously observed response to tetragastrin, a similar dose of G-CA was administered intravenously. Since G-CA is comprised of tetragastrin and cholic acid in an approximate weight ratio of 3:2 (formula weights of tetragastrin and cholic acid are 597 and 408, respectively), the dose injected in the first instance was 40μg kg^{-1} (i.e. equivalent to 25μg kg^{-1} tetragastrin alone). This was successful in every case (n=4) in evoking an increase in gastric acid secretion for which both the mean peak increase of 0.62 ± 0.22μmol $15min^{-1}$ and the mean cumulative increase in acidity over the first hour following administration of 1.45 ± 0.14μmol hr^{-1} were statistically significant (P=0.011 and P=0.001, respectively) (results not tabulated).

However, it was later considered necessary to reduce this intravenous dose to a minimum effective dose of 15μg kg^{-1} (for reasons explained in Section 3.1.3); nevertheless, in the experiments in which 15μg kg^{-1} was administered, a significant increase in both the mean peak response (0.49 ± 0.13μmol $15min^{-1}$; P=0.002; n=5), and the mean cumulative response (1.10 ± 0.73μmol hr^{-1}; P=0.027; n=5) was present (Table 3-5). There was a divergence in the collection period, after the intravenous injection, in which the peak response occurred. In 3 rats, it arose in the first collection period (i.e. the same one in which the injection was given), whereas in 2 rats, it was presented in the second and in the third collection period. The increase in total gastric acidity above baseline in response to intravenous G-CA lasted for between 15 and 30 minutes in 3 out of 5 rats. Since the gastric acid levels did not return to baseline, but remained slightly elevated, in 2 rats, the duration of response was difficult to quantify.

Interestingly, the mean increases in total gastric acidity evoked by 15μg kg^{-1} G-CA (I.V.) were very similar in magnitude with the rise in gastric acidity obtained in response to 20μg kg^{-1} intravenous tetragastrin (Table 3-1) (mean peak absolute increase in acidity of 0.53 ± 0.25μmol $15min^{-1}$, *c.f.* 0.49 ± 0.13μmol $15min^{-1}$ elicited by 15μg kg^{-1} I.V. G-CA, and mean cumulative increase in acidity of 1.38 ± 0.84μmol hr^{-1}, *c.f.* 1.10 ± 0.73μmol hr^{-1} elicited by 15μg kg^{-1} I.V. G-CA). A two sample t-test determined that neither the peak responses nor the cumulative responses to tetragastrin and to G-CA were significantly different in these two sets of experiments (P=0.77 and P=0.56, respectively).

A typical experiment is illustrated in Figure 3-5, whereby 15μg kg^{-1} G-CA was administered intravenously (arrow), following attainment of a steady baseline, 45 minutes after sampling had begun. A peak increase in gastric acid secretion in response to G-CA was observed in the first collection period containing administration (that is, between 0 and 15 minutes after bolus injection), and total acidity was seen to rise to 0.43μmol $15min^{-1}$ above control values. The cumulative increase in gastric acid levels over the hour following injection was 1.13μmol hr^{-1} (although the response could reasonably be considered to last for only 30 minutes, since a new elevated baseline was reached after 45 minutes).

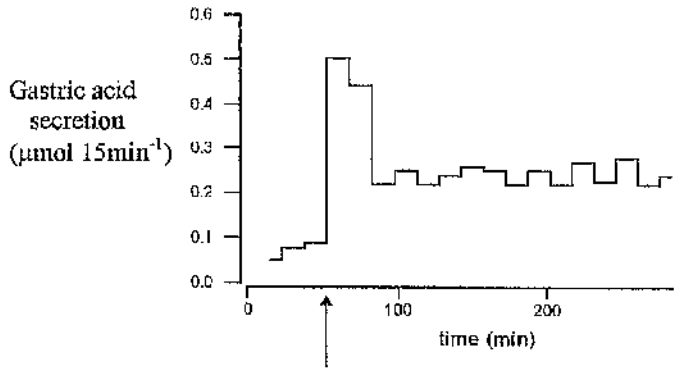


Fig. 3-5: The effect of intravenous G-CA (15μg kg^{-1}) (arrow) on gastric acid secretion in the anaesthetised rat (rat 5 in Table 3-5). Figure illustrates the initial results for this particular experiment in order to show the biological activity of G-CA. The full record is given in Figure 3-6.

The reproducibility of the response was tested with a second intravenous injection of G-CA (15μg kg^{-1}, I.V.) administered 180 minutes after settling of the baseline following the first intravenous injection of G-CA in 5 rats. This was to determine whether the response to the second intravenous injection differed significantly from the response to the first, thereby allowing us to compare our results from these experiments with those obtained in later experiments in which G-CA was administered intra-ileally in-between the intravenous infusions of G-CA.

The initial intravenous injection of G-CA (15μg kg^{-1}) elicited a significant mean peak increase in acidity of 0.49 ± 0.13μmol $15min^{-1}$ (P=0.002) above baseline, and a mean cumulative rise in gastric acid secretion of 1.10 ± 0.73μmol hr^{-1} (P=0.027), which may be compared with the subsequent administration of an equivalent dose of G-CA, which resulted in a mean peak increase in acidity of 0.65 ± 0.28μmol $15min^{-1}$ (P=0.007) and a cumulative increase of 1.46 ± 0.84μmol hr^{-1} (P=0.018) (Table 3-5). Application of a paired t-test demonstrated that the absolute peak increase and the cumulative increase in acidity above baseline elicited by the first and second injections of G-CA were not significantly different (P=0.35 and P=0.57, respectively).

Table 3-5: Peak and cumulative (cum.) increases in gastric acid secretion in response to I.V. tetragastrin-cholic acid conjugate (G-CA) (15μg kg^{-1}) as a first and final procedure.

Rat	Initial I.V. G-CA		Second I.V. G-CA	
	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})
1	0.46	1.18*	0.54	1.43*
2	0.73	2.25*	0.54	1.19
3	0.43	0.43	1.13	2.89
4	0.42	0.53	0.43	0.72
5	0.43	1.13	0.61	1.08
Mean ± S.D.	0.49±0.13 (P=0.002)	1.10 ± 0.73 (P=0.027)	0.65±0.28 (P=0.007)	1.46±0.84 (P=0.018)

P-values denote statistical significance for compartson with baseline.

* *denotes establishment of new baseline which was elevated compared with that at start of experiment.*

The results of a full typical experiment are illustrated in Figure 3-6, in which 15μg kg^{-1} G-CA was injected 45 minutes after collections began (first arrow), and a settling of the baseline had been attained (second arrow). The initial 15μg kg^{-1} dose elicited a peak increase of 0.43μmol $15min^{-1}$ above baseline in the first collection period (that is, between 0 and 15 minutes after administration), and the increased response lasted for a total of 30 minutes before returning to control levels, resulting in a cumulative enhanced response of 1.13μmol hr^{-1}. The response to the second intravenous injection was similar in both magnitude and duration. A peak increase of 0.61μmol in the first collection period, and a cumulative rise of 1.08μmol hr^{-1} were recorded. Again, the duration of increased acidity above original levels was 30 minutes.

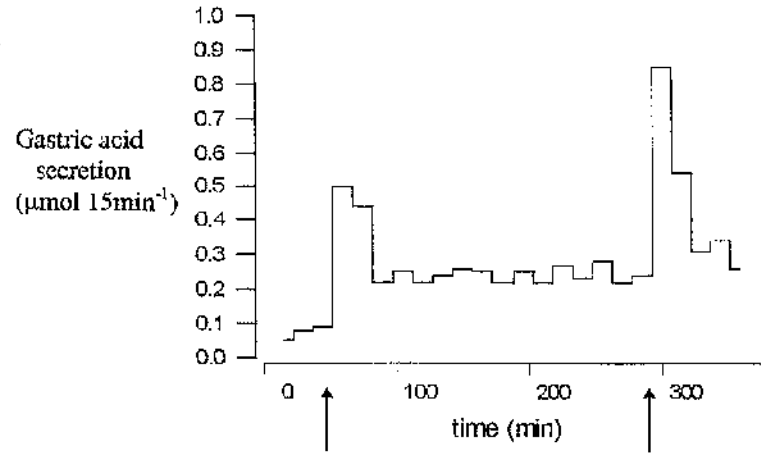


Fig. 3-6: The effect of intravenous G-CA (15μg kg^{-1}, first and second arrow) on gastric acid secretion in the anaesthetised rat (rat 5 in Table 3-5). The initial part of this figure is shown in Fig. 3-5.

3.1.3 Gastric acid secretion in response to ileally-administered tetragastrin-cholic acid conjugate (G-CA)

3.1.3(i) *Ileal administration of G-CA (1600μg kg^{-1})*

The establishment of an unequivocal increase in acidity in response to intravenously administered G-CA prompted us to proceed with our experiments to test the ability of ileally-administered G-CA to enhance gastric acid secretion.

In these experiments, a similar experimental protocol to that carried out in the control experiments using tetragastrin was followed. Therefore, we initially administered 40μg kg^{-1} I.V. G-CA, followed by 1600μg kg^{-1} ileal G-CA for a period of 180 minutes, followed finally by a subsequent injection of G-CA (40μg kg^{-1}, I.V.). This procedure was carried out on four rats, and produced the following results (Figure 3-7A-D).

Figure 3-7A illustrates the results obtained for one of these rats. Intravenous administration of G-CA (40μg kg^{-1}) (first arrow) elicited a peak increase in gastric acidity of 0.46μmol $15min^{-1}$ above baseline over the first 15 minutes following injection. The response lasted for a total of 15 minutes before settling at an elevated baseline. Ileal administration of 1600μg kg^{-1} G-CA (in 1.0ml isotonic saline) (second arrow) gave a surprising response. In the first period after administration, gastric acid levels rose to 0.17μmol $15min^{-1}$ above baseline; a peak response of 0.28μmol $15min^{-1}$ was observed after 60 minutes. The enhanced response was still present at 135 minutes, when the total cumulative increase in gastric acid secretion above basal levels was 1.89μmol $135min^{-1}$. A second intravenous injection of G-CA (40μg kg^{-1}) (third arrow) was then administered, and resulted in a peak increase in gastric acid levels above baseline of 0.31μmol $15min^{-1}$ in the first 15 minutes. The response to G-CA (I.V.) lasted for 30 minutes, giving an increased total acidity of 0.61μmol overall.

The results of a second similar experiment (Fig. 3-7B) demonstrated that intravenously administered G-CA (40μg kg^{-1}) (first arrow) resulted in an increase in gastric acid levels above baseline which, in this case, was unusually high, with a peak rise of 0.93μmol $15min^{-1}$ in the first collection period. The elicited response lasted for a duration of 30 minutes, and the total increase in acid levels was 1.13μmol $15min^{-1}$. Ileal administration of G-CA (1600μg kg^{-1} in 1.0ml isotonic saline) (second arrow) did not result in an increase in acidity; rather, gastric acid levels over the 180 minute duration were observed to drop by a total of 6.76μmol. In order to confirm that the preparation was still sensitive to G-CA, a second

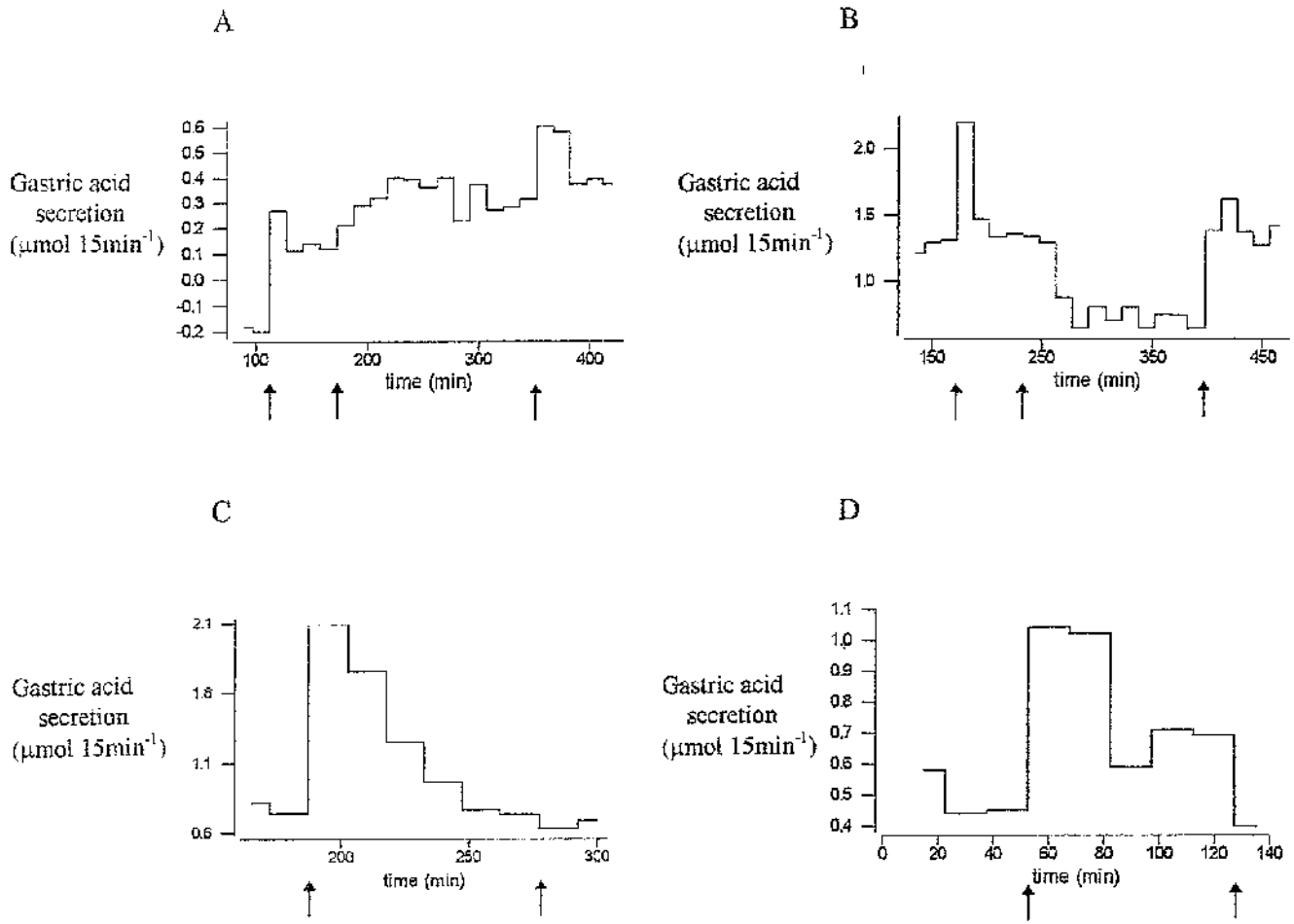


Fig.3-7: The effect of intravenous G-CA (40μg kg^{-1}, first arrow (A-D) and third arrow (A-B)) and intra-ileal G-CA (1600μg kg^{-1}, second arrow(A-D)) on gastric acid secretion in the anaesthetised rat. (N.B. Scales are not identical.)

intravenous injection of G-CA (40μg kg^{-1}) (third arrow) was administered, which resulted in a similar peak increase in acidity as that elicited by the first intravenous injection. The peak increase of 0.92μmol $15min^{-1}$ was observed in the second collection period, that is, between 15 and 30 minutes after injection. However, the duration of the response, and a value for the cumulative response were difficult to quantify since acidity appeared to settle down at a higher gastric acid level, that is, the baseline appeared to shift upwards.

The conflicting results observed in these two experiments were puzzling. It was noted that the gastric acid levels prior to ileal administration of G-CA were very much lower in the rat in which ileally-administered G-CA was effective in evoking increased levels of gastric acid secretion than in the rat in which ileal G-CA was ineffective (0.12μmol $15min^{-1}$ and 1.34μmol $15min^{-1}$, respectively). However, why the initial baseline should influence the response to ileal G-CA, especially when intravenous injection of G-CA evoked a normal response, is difficult to explain.

Similar procedures were attempted on a further two rats (Figs. 3-7C and 3-7D). Intravenous administration of G-CA (40μg kg^{-1}) (first arrow) elicited an increase in gastric acid levels in the first 15 minutes after injection to a peak value of 1.31μmol $15min^{-1}$, and 0.60μmol $15min^{-1}$, in rats 3 and 4, respectively. The response remained raised above baseline for 45 minutes in rat 3, and for 30 minutes in rat 4, resulting in a total increase in acidity of 2.76μmol (rat 3) and 1.17μmol (rat 4). However, ileal administration of 1600μg kg^{-1} G-CA (in 1.0ml isotonic saline) (second arrow) appeared to have a detrimental effect on both rats, with rat 4 dying only 15 minutes after introduction of ileal G-CA. Rat 3 survived for only 30 minutes following administration of ileal G-CA.

3.1.3(ii) *Ileal administration of G-CA (600μg kg^{-1})*

As a precaution, we decided to reduce considerably the dose of the intravenously and ileally-administered drugs in an attempt to ensure the viability of the rat, thereby reducing the risk of incomplete experiments. We therefore adopted a new protocol using reduced doses in which 15 or 20μg kg^{-1} G-CA was intravenously injected prior to and following the administration of 600μg kg^{-1} ileal G-CA, and the changes in total gastric acidity were measured in the same way. Experiments were

carried out on 7 rats in total, of which 5 provided evidence to suggest that ileally-administered G-CA (600μg kg^{-1}), *per se*, evoked an unequivocal increase in gastric acidity (Table 3-6). Figure 3-8 illustrates the results of such an experiment.

Table 3-6: Peak and cumulative (cum.) increases (inc.) in gastric acid secretion in response to ileally-administered tetragastrin-cholic acid conjugate (G-CA) (600μg kg^{-1} in 1.0ml saline) over 180 minute period, and to intravenous G-CA (15μg kg^{-1}) as a first and final procedure.

Rat	Initial I.V. G-CA		Ileal G-CA	Second I.V. G-CA	
	Peak increase (μmol 15min^{-1})	Cum. increase (μmol hr^{-1})	Cumulative increase (μmol 180min^{-1})	Peak increase (μmol 15min^{-1})	Cum. increase (μmol hr^{-1})
1	0.30	0.48	1.34	0.30	0.49
2	0.86	1.78*	0.83	0.67	1.62
3	0.57	1.49	2.01	0.90	2.20
4	0.84	1.48	7.66	0.93	1.49
5	3.21†	8.11†	3.62	0.79	1.34
6	0.17	0.28	-1.28	1.50	4.41
7	0.73	2.07*	0.25	0.81	1.63*
Mean ⊗ (rats 1-5)	0.64±0.26 (P=0.017)	1.31±0.57 (P=0.019)	2.80 ± 2.17 (P=0.045)	0.72±0.26 (P=0.003)	1.43±0.62 (P=0.007)
Mean ⊗ (rats 6-7)	0.45	1.18	-0.52	1.15	3.02

* *denotes establishment of new baseline which was elevated compared with that at start of experiment.*

† *denotes value omitted from analysis due to disproportionately large value (see text for details)*

⊗ *mean responses are grouped depending on the effectiveness of ileally-administered in inducing elevation of gastric acid secretion (see text for details)*

Intravenous injection of G-CA (15μg kg^{-1}) (first arrow) led to a peak increase in gastric acidity of 0.57μmol $15min^{-1}$ in the fourth collection period, although a strong response was also present between 15 and 30 minutes after injection (0.44μmol $15min^{-1}$). The increase in gastric acidity lasted for 75 minutes, and the cumulative increase in acidity over the hour following injection was 1.49μmol hr^{-1}. Administration of ileal G-CA (600μg kg^{-1} in 1.0ml isotonic saline) (second arrow) was then undertaken. The peak increase in acidity of 0.59μmol $15min^{-1}$ occurred

between 75 and 90 minutes after administration, though a sizeable increase in acid levels of 0.37μmol 15min^{-1} was noted 15 to 30 minutes following addition of G-CA.

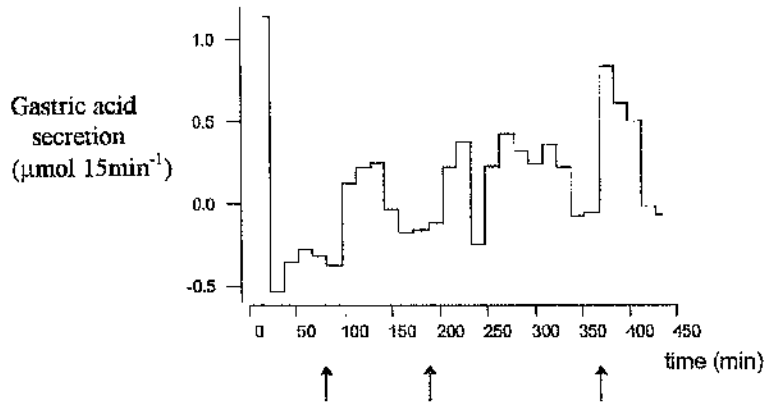


Fig. 3-8: The effect of intravenous G-CA (15μg kg^{-1}, first and third arrow), and ileally-infused G-CA (600μg kg^{-1}, second arrow) on gastric acid secretion in the anaesthetised rat (rat 3 in Table 3-6).

The gastric acid levels maintained their increased level above baseline for 150 minutes before falling back to control levels. A transitory drop in gastric acid levels of 0.62μmol 15min^{-1} back to the original baseline was recorded 45 minutes after ileal administration of G-CA had been conducted, lasting no longer than 15 minutes; however, we are unsure as to why this occurred. Nevertheless, a cumulative increase in acidity of 2.01μmol 180min^{-1} was found in response to the presence of G-CA in the ileum. Finally, an injection of G-CA (15μg kg^{-1}) (third arrow) intravenously, elicited a peak response of 0.90μmol 15min^{-1} in the first collection period. The increased response above baseline lasted for a duration of 45 minutes, and gave a cumulative increase in total acidity of 2.20μmol hour^{-1}.

Analysis of the responses obtained in these 5 experiments provided the following results. A significant mean peak increase above baseline in total acidity of 0.64 ± 0.26μmol 15min^{-1} (P=0.017) elicited by 15μg kg^{-1} G-CA (I.V.) was observed, as was a cumulative increase in acid levels above baseline of 1.31 ± 0.57μmol hr^{-1} (P=0.019). The results of one experiment in which the peak and the cumulative increases in gastric acid secretion were 3.21μmol 15min^{-1} and 8.11μmol hr^{-1},

respectively, were omitted from the two sample t-test, since they would have biased the results by a disproportionately large amount. The peak value obtained generally occurred in the first or second collection period, and the response typically lasted for between 30 and 60 minutes after administration. Ileally-administered G-CA (600μg kg^{-1}) resulted in a significant mean peak increase in acidity of 0.71 ± 0.43μmol $15min^{-1}$ (P=0.002) and a total increase in gastric acid levels, over a period of 180 minutes, of 2.80 ± 2.17μmol (P=0.045). Peak acid levels were observed to occur between 75 and 105 minutes after ileal infusion. However, two rats did demonstrate a peak response in the 11th and the 12th collection period (165 minutes and 180 minutes after administration, respectively). Nevertheless, strong responses were observed prior to this in the first collection period (the 15 minutes containing the ileal administration). Subsequent intravenous injection of G-CA (15μg kg^{-1}) evoked elevated acid levels of similar magnitude to those elicited by the first intravenous injection. A mean peak increase of 0.72 ± 0.26μmol $15min^{-1}$ (P=0.003), and a mean cumulative increase of 1.43 ± 0.62μmol hr^{-1} (P=0.007) were attained. The peak gastric acid values were typically obtained in the period of injection of G-CA, and the acid levels appeared to remain elevated above baseline for a duration of 30 to 45 minutes. Furthermore, a paired t-test demonstrated that, similar to the experiments in which a first and a second I.V. injection of G-CA (15μg kg^{-1}) were administered in the absence of ileally-administered G-CA, the evoked peak and cumulative responses to intravenous injection of G-CA prior to and following ileal administration of G-CA in this case were not significantly different (P=0.63 and P=0.51, respectively). This suggests that the presence of G-CA in the ileum did not enhance the elicited increase in acidity in response to the second intravenous injection of G-CA.

Of the experiments carried out in this series (n=7), two rats failed to show any evidence of an enhanced gastric acid secretion following administration of ileal G-CA, even though intravenously injected G-CA (15μg kg^{-1}) evoked increases in gastric acid output as in previous experiments. Figure 3-9 demonstrates the outcome of one of these experiments. As can be seen, intravenous injection of G-CA (first arrow) elicited an increase in acidity to a peak value of 0.73μmol $15min^{-1}$

(N.B. baseline lies below zero at a gastric acidity of -0.35μmol 15min^{-1}). This was seen to occur in the first collection period. The actual duration of the response was difficult to define, as was the cumulative increase in acidity, since there appeared to be a substantial decline in acid levels to zero after 30 minutes; however, this was followed by a steady rise in acidity over a period of 60 minutes to a new control level of 0.33μmol 15min^{-1}.

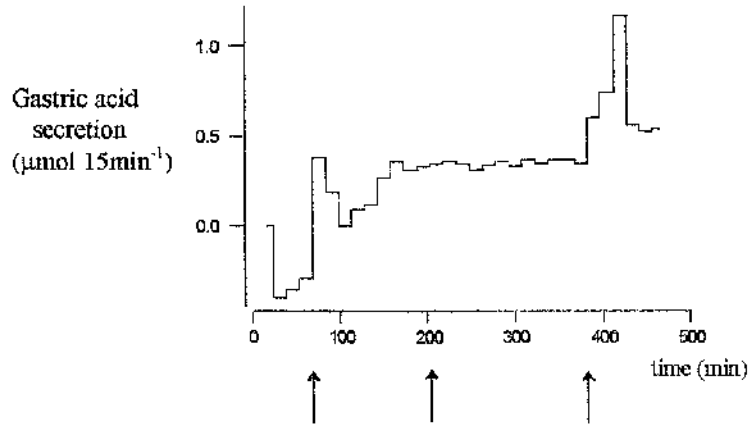


Fig. 3-9: The effect of intravenous G-CA (15μg kg^{-1}, first and third arrow), and ileally-infused G-CA (600μg kg^{-1}, second arrow) on gastric acid secretion in the anaesthetised rat (rat 7 in Table 3-6).

Following administration of ileal G-CA (600μg kg^{-1}) (second arrow), gastric acid levels remained steady at between 0.31 and 0.37μmol 15min^{-1} over a 180 minute period. When this was compared with the baseline taken to be the mean of the three collection periods preceding the intra-ileal injection, this amounted to a calculated total increase in acidity of 0.25μmol 180min^{-1} which, when averaged out over each 15 minute sampling period, resulted in an increase of only 0.02μmol 15min^{-1}. This was assumed to be negligible. A second intravenous injection of G-CA (15μg kg^{-1}) (third arrow) produced a comparable increase in acidity with the initial injection, although the peak value of 0.81μmol 15min^{-1} occurred in the 3rd collection period, rather than the first. The response was considered to last for 45 minutes, totalling a cumulative increase in gastric acid levels of 1.63μmol hr^{-1}. The viability of the preparation was therefore confirmed.

A noteworthy result was present in the other rat in this group of two, in that although ileally-administered G-CA was ineffective in evoking gastric acid secretion above basal levels, the response to the second intravenous injection of G-CA elicited an increase in total acidity greatly potentiated compared with the response to the first. Figure 3-10 illustrates the results obtained. Initial intravenous injection of G-CA (15μg kg^{-1}) (first arrow) was effective in producing a peak increase in total acidity, though only of 0.17μmol $15min^{-1}$, in the period of

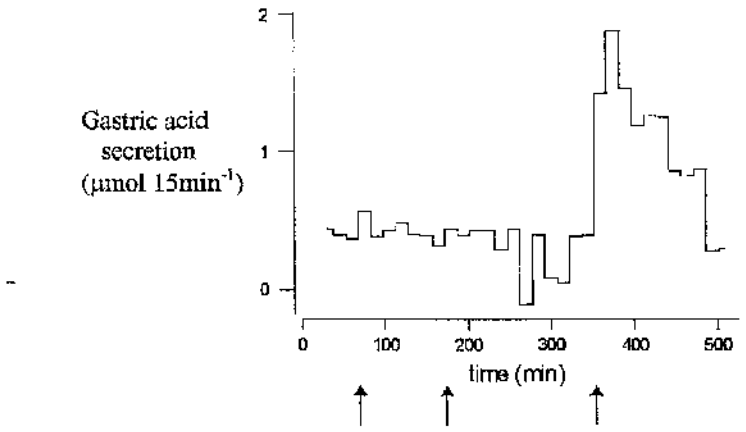


Fig. 3-10: The effect of intravenous G-CA (15μg kg^{-1}, first and third arrow), and ileally-infused G-CA (600μg kg^{-1}, second arrow) on gastric acid secretion in the anaesthetised rat (rat 6 in Table 3-6).

administration. Gastric acid levels remained notionally elevated above baseline for 60 minutes, culminating in a small total increase in gastric acid secretion of 0.28μmol hr^{-1}. Following this, ileal administration of G-CA (600μg kg^{-1}) was carried out (second arrow), and over a period of 3 hours, changes in gastric acid secretion amounted to a drop of 1.28μmol $180min^{-1}$ below pre-administration acid levels. A surprising result came in response to a second intravenous injection of G-CA (15μg kg^{-1}), 180 minutes after ileal administration (third arrow). A relatively very large peak increase in gastric acid levels of 1.50μmol $15min^{-1}$ was recorded in the period after injection (between 15 and 30 minutes after intravenous infusion). A cumulative increase in gastric acid secretion above baseline of 4.41μmol hr^{-1} was attained and gastric acid levels remained greatly elevated above pre-injection value for 135 minutes in total. Therefore, the peak response and the

cumulative response to the second injection (I.V.) were 8.82 and 15.75 times larger, respectively, than those in response to the first injection. These were exceptional in that they were very much larger than had been observed in any of the other experiments in the course of this study. It could be suggested that the greatly enhanced acid secretion in response to the second intravenous injection may have arisen due to potentiation by the presence of subthreshold doses in the circulation emanating from the ileal G-CA, although if that were the case, the noticeable lack of any response over the period of three hours prior to intravenous injection is somewhat surprising.

Nonetheless, the outcome, as seen in two rats, in which the animal remained unresponsive to the effects of ileally-administered G-CA provided us with confidence that our method of administration (via an ileal cannula) was not in itself accountable, for example, by leakage of G-CA, for the increases in gastric acid levels elicited by ileally-infused G-CA in the other five experiments. This was further supported by our control experiments in which ileal tetragastrin was unsuccessful in increasing gastric acid levels above baseline, even though intravenously administered tetragastrin evoked a sizeable augmentation of acidity.

The application of statistical analysis of the results obtained from the experiments in which ileal G-CA was ineffective in inducing enhanced gastric acid levels was not considered appropriate with a group of only two, and the variation in response was considerable. Therefore, the mean responses were calculated, and were as follows. Intravenous administration of G-CA (15μg kg^{-1}) resulted in a mean peak increase of 0.45μmol $15min^{-1}$ above control gastric acid levels, a value not dissimilar to that obtained in the five rats in which ileal G-CA was effective in enhancing gastric acid secretion. In both rats, the peak was noted to occur in the first 15 minutes after administration. A mean cumulative acidity of 1.18μmol hr^{-1} was calculated although, due to the gastric acidity settling at a higher level than the original baseline, the actual duration of response was difficult to define. Following ileal injection of the G-CA (600μg kg^{-1}), a mean decrease in gastric acidity of 0.52μmol $180min^{-1}$ was observed. We therefore concluded that ileal injection of G-CA had been ineffective in inducing any discernible increase in gastric acid

levels. A second injection of G-CA (15μg kg^{-1}), intravenously, enabled us to demonstrate the retained sensitivity of the animal to the effects of G-CA. An increase in the mean peak acidity, occurring in the second collection period, of 1.15μmol $15min^{-1}$ above baseline (2.56 times greater than the response to the initial I.V. G-CA injection), and a total cumulative acidity of 3.02μmol hr^{-1} (2.56 times greater than that initiated by the initial injection) was demonstrated.

3.1.3(iii) *Ileal administration of tetragastrin (3000μg kg^{-1}) subsequent to intra-ileal G-CA (600μg kg^{-1})*

Based on our observations that ileally-introduced G-CA appeared able to cross the wall of the small intestine and enter the bloodstream, thereby increasing gastric acid secretion, we were interested to determine whether any changes caused by G-CA to the small intestine were long lasting and non-specific. To this end, we decided to carry out a series of experiments similar to those described previously, whereby the infusion of ileal G-CA (600μg kg^{-1}) was followed 180 minutes later by ileal administration of tetragastrin (3000μg kg^{-1} in 1.0ml isotonic saline). The principle behind this protocol lay with the premise that when testing for a physiological effect, a modest dose was administered (in this case, 600μg kg^{-1} G-CA), whereas when testing for the absence of an effect, a very high dose was used (in this case, 3000μg kg^{-1} tetragastrin). The dose of G-CA administered was equivalent to that introduced in previous experiments, where there was evidence of an elicited physiological response, and the dose of tetragastrin used was similar to earlier studies where there was no evidence of a response being evoked.

The outcome of a typical experiment is illustrated over (Fig. 3-11). Intravenously administered G-CA (15μg kg^{-1}, first arrow) elicited a peak increase in gastric acid secretion of 0.39μmol $15min^{-1}$ above control levels in the collection period in which injection was carried out. The gastric acid levels remained elevated for 30 minutes, resulting in a cumulative increase in total gastric acidity of 0.75μmol hr^{-1}, before falling to a level slightly below pre-injection levels.

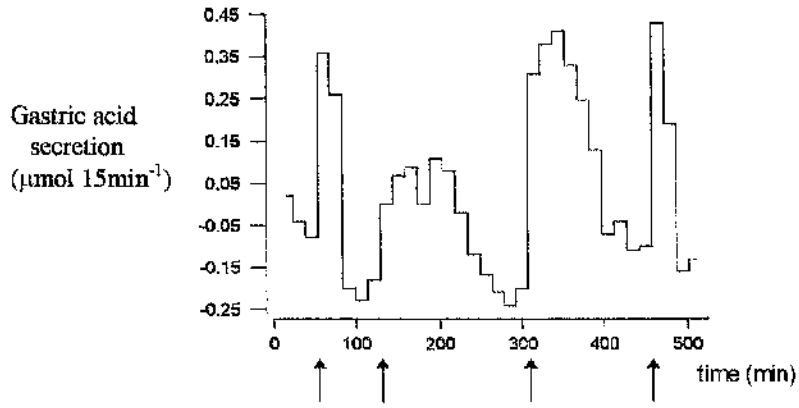


Fig. 3-11: The effect of intravenous injection of G-CA (15μg kg^{-1}, first and fourth arrows), and ileal infusion of G-CA (600μg kg^{-1}, second arrow), followed 180 minutes later by ileally-infused tetragastrin (3000μg kg^{-1}, third arrow) on gastric acid secretion in the anaesthetised rat (rat 6 in Table 3-7).

Following return of gastric acid levels to a steady level, infusion of G-CA (600μg kg^{-1}) into the ileum was performed, and resulted in a peak increase in acid secretion of 0.31μmol 15min^{-1} in the 5th collection period (between 60 and 75 minutes after administration of ileal G-CA). Nonetheless, a sizeable increase in total acidity was presented in the period of infusion of 0.20μmol 15min^{-1}. Elevated levels were maintained above basal levels for 120 minutes, culminating in a total increased level of 1.87μmol 180min^{-1}. In order to determine whether the increased response above basal levels to ileal G-CA was mediated via a non-specific and long-lasting change to the ileal ultrastructure, we gave an ileal infusion of tetragastrin (3000μg kg^{-1}) 180 minutes after ileally-infused G-CA. This resulted in a considerable increase in gastric acid secretion of 0.53μmol 15min^{-1} in the period of adminstration. The increased total acidity rose steadily to a peak value of 0.63μmol 15min^{-1} in the third collection period, that is, between 30 and 45 minutes after injection. The response lasted for a duration of 90 minutes before falling to a slightly elevated baseline. The cumulative increase in gastric acid secretion was 3.13μmol 180min^{-1}, i.e. larger than that evoked by 600μg kg^{-1} G-CA. Finally, we carried out an identical intravenous injection of G-CA to the first, which resulted in a response of comparable size and duration. A peak increase in gastric acid levels of 0.51μmol 15min^{-1} was attained in the first collection period, and gastric acid

levels remained elevated above baseline for 30 minutes, resulting in an overall cumulative response of 0.78μmol hr^{-1}.

Seven such experiments were carried out. The mean values (Table 3-7) demonstrated that an initial injection of G-CA (15μg kg^{-1}, I.V.) resulted in a significant peak increase in gastric acid levels above baseline of 0.57 ± 0.24μmol $15min^{-1}$ (P=0.001). The peak value obtained generally occurred in the first or second collection period, and the response typically lasted for between 30 and 45 minutes before returning to baseline. This elevation of gastric acid levels above baseline resulted in a mean cumulative response of 1.09 ± 0.52μmol hr^{-1}, which was statistically significant (P=0.004).

Table 3-7: Peak and cumulative (cum.) increases in gastric acid secretion in response to ileally-administered tetragastrin-cholic acid conjugate (G-CA) (600μg kg^{-1} in 1.0ml saline) over 180 minute period, subsequent ileally-administered tetragastrin (G4) (3000μg kg^{-1}) over 180 minute period, and intravenous G-CA (15μg kg^{-1}) as a first and final procedure.

Rat	Initial I.V. G-CA		Ileal G-CA	Ileal G4	Second I.V. G-CA	
	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})	Cum. increase (μmol $180min^{-1}$)	Cum. increase (μmol $180min^{-1}$)	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})
1	0.47	1.55	1.40	5.25	0.34	0.80
2	1.03	1.45	9.89†	6.01	1.62	2.27†
3	0.49	1.67	0.84	2.30	0.68	1.61
4	0.77	2.87*†	0.90	0.94	0.68	0.96
5	0.38	0.56	4.70†	1.98	0.64	0.98
6	0.39	0.75	1.87	3.13	0.51	0.78
7	0.47	0.58	1.53	1.77	0.46	0.61
Mean ± S.D.	0.57±0.24 (P=0.001)	1.09±0.52 (P=0.004)	1.31±0.44 (P=0.003)	3.05±1.89 (P=0.006)	0.70±0.42 (P=0.005)	1.15±0.59 (P=0.003)

P-values denote statistical significance for comparison with baseline.

* *denotes establishment of new baseline which was elevated compared with that at start of experiment.*

† *denotes value omitted from analysis due to disproportionately large value (see text for details)*

The result of one experiment was omitted from the analysis; a cumulative response of 2.87μmol hr^{-1} was recorded which deviated from the normal distribution of responses. However, application of the Wilcoxon statistical test (in which the

median of the sample is tested, thereby allowing the population to deviate from a normal distribution) confirmed the significance of the elevated gastric acid levels in response to intravenous tetragastrin; a cumulative response with an estimated median of 1.18μmol hr^{-1} (P=0.022) was found.

In all seven experiments, without exception, ileal infusion of G-CA (600μg kg^{-1}), subsequent to intravenous G-CA, produced an increase in gastric acid levels. A mean peak increase in total acidity of 0.53 ± 0.42μmol $15min^{-1}$ was observed, which was statistically significant, with respect to baseline (P=0.015), although its period of occurrence was variable (results not tabulated). In one rat, the peak response was presented in the period of administration; in another it appeared in the third. A further two rats demonstrated the peak in the fifth collection period, whereas it was achieved in the sixth and in the seventh collection period in two others. Finally, in one rat, the peak increase in gastric acid secretion occurred in the twelfth collection period (Fig. 3-12). Nonetheless, in every case, a sizeable increase in gastric acid secretion was achieved in the period of administration followed by a typically steady increase to peak levels.

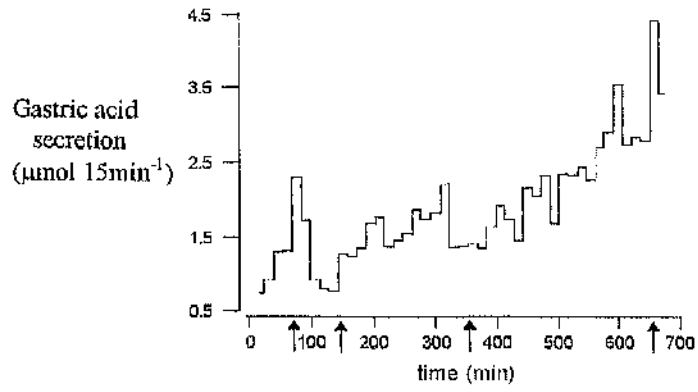


Fig. 3-12: The effect of intravenous injection of G-CA (15μg kg^{-1}, first and fourth arrows), and ileal infusion of G-CA (600μg kg^{-1}, second arrow), followed 210 minutes later by ileally-infused tetragastrin (3000μg kg^{-1}, third arrow) on gastric acid secretion in the anaesthetised rat (rat 2 in Table 3-7).

The duration of response was shown to be somewhat more consistent amongst individual rats, ranging between 105 and 135 minutes. In only one rat was a slightly more long lasting elevation of gastric acid levels of 180 minutes observed (Fig. 3-12). Thus, a mean cumulative increase of 1.31 ± 0.44μmol $180min^{-1}$ was found to be statistically significant (P=0.003). The analysis did not include the results of two experiments in which the cumulative increases in gastric acidity were 4.70 and 9.89μmol $180min^{-1}$, since this would have biased the results by a disproportionately large amount. However, application of the Wilcoxon statistical test to all the data confirmed that the cumulative increase of gastric acid, compared with baseline, presented an estimated median value of 1.79μmol $180min^{-1}$, which was indeed statistically significant (P=0.022).

The administration of ileal tetragastrin (3000μg kg^{-1}), 180 minutes after infusion of ileal G-CA, caused, unequivocally, a large increase in gastric acid secretion, compared with baseline. Over a period of 3 hours following infusion, total gastric acidity evoked above baseline amounted to a mean cumulative increase of 3.05 ± 1.89μmol $180min^{-1}$, which was found to be statistically significant (P=0.006). Also recorded was a significant mean peak increase in gastric acid secretion of 0.71 ± 0.67μmol $15min^{-1}$ (P=0.030) although, as for the peak response to ileally-infused G-CA, the period in which it occurred was variable. In five experiments, the peak occurred in either the first (n=1), second (n=1), third (n=2) or fourth (n=1) collection period; however, in two other experiments, the peak increase was evoked in the seventh and in the seventeenth period after injection. With the exception of two rats, a sizeable increase in gastric acid secretion was elicited in the period of ileal infusion of tetragastrin, which steadily increased to peak values. In the exceptions, a response above baseline was not evoked until the second and the third collection periods after infusion. The response above pre-infusion levels generally lasted for between 90 and 105 minutes; however, a notable exception was the rat in which gastric acid levels remained elevated above control value for 255 minutes (Fig. 3-12). This was the same rat which had presented the peak increase in total gastric acidity in the seventeenth period in response to ileal tetragastrin, and the peak increase in response to ileal G-CA in the twelfth period.

The viability of the preparation in the final stages of the investigation was confirmed when, on injection of G-CA (15μg kg^{-1}, I.V.) as the final procedure, a peak response of 0.70 ± 0.42μmol $15min^{-1}$ and a cumulative response of 1.15 ± 0.59μmol hr^{-1} were presented and statistically significant, with respect to baseline (P=0.005 and P=0.003, respectively). The observed cumulative response of one animal to intravenous G-CA as the final procedure was not included in the statistical analysis, since it demonstrated an increase in gastric acid secretion, outwith the normal distribution of results, of 2.27μmol hr^{-1}. However, application of the Wilcoxon test to all the data confirmed a significant increase in gastric acid secretion, with an estimated median value of 0.96μmol hr^{-1}. (P=0.022). Furthermore, there was no significant difference between either the peak or the cumulative responses of the first and second intravenous infusions of G-CA (P=0.21 and P=0.57, respectively), which suggest that, at this late stage of the investigation, the presence of ileal tetragastrin did not further augment the response.

3.1.3(iv) *Ileal administration of the components of G-CA (600μg kg^{-1})*

It was necessary to determine whether the introduction of the components making up G-CA, that is, tetragastrin and cholic acid, into the ileum would evoke a similar increase in gastric acidity as that seen when G-CA (600μg kg^{-1}) was administered ileally. Unfortunately, cholic acid is quite insoluble in isotonic saline, and we were therefore obliged to resort to an alternative, glycocholic acid, which was easily dissolvable. A solution of tetragastrin and glycocholic acid (in total, 600μg kg^{-1}) was made up, so that the ratio of peptide and bile acid in solution were identical to those of the ileally-infused conjugate (approx. 3:2 ratio by weight). Therefore, the 600μg kg^{-1} solution was comprised of 356.4μg kg^{-1} tetragastrin (59.4%), and 243.6μg kg^{-1} glycocholic acid (40.6%). This solution was then infused subsequent to an initial intravenous injection of G-CA (15μg kg^{-1}). An example of the outcome of such an experiment is illustrated (Fig. 3-13). Initially, an intravenous injection of G-CA (15μg kg^{-1}) (first arrow) was administered to ensure the animal was responsive. This was shown to evoke increased secretion of gastric acid to a peak value of 0.42μmol $15min^{-1}$ above baseline in the second collection period

after administration. The acid levels remained elevated above control levels for a duration of 30 minutes before returning to their original value, amounting to a cumulative increase in acidity of 0.64μmol hr^{-1} above baseline.

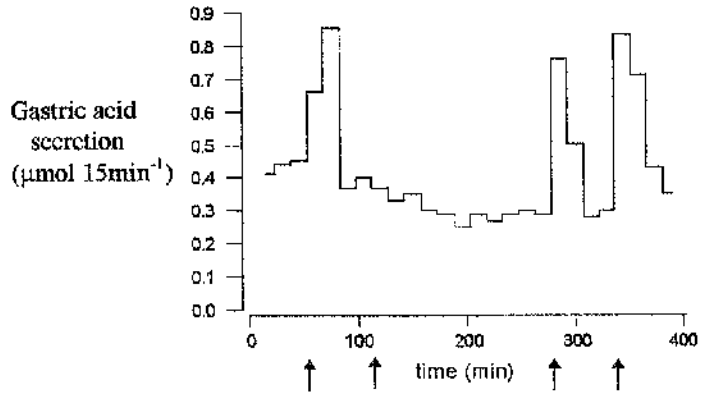


Fig. 3-13: The effect of intravenous injection of G-CA (15μg kg^{-1}, first and third arrows), ileal infusion of tetragastrin:glycocholic acid (3:2 ratio, total 600μg kg^{-1}, second arrow), and intravenous injection of tetragastrin:glycocholic acid (3:2 ratio, total 15μg kg^{-1}, fourth arrow) on gastric acid secretion in the anaesthetised rat (rat 4 in Table 3-8).

Following the return of gastric acid levels to their original value, ileal infusion of the solution containing tetragastrin and glycocholic acid (600μg kg^{-1}, in a 3:2 ratio) was carried out (second arrow), and demonstrated that, over a 180 minute period, there was little evidence of any increase in gastric acid levels. A cumulative decrease of 0.18μmol $180min^{-1}$ with respect to the baseline was actually presented. A second intravenous injection of G-CA (15μg kg^{-1}) (third arrow) was then administered, resulting in a peak increase in acidity of 0.48μmol $15min^{-1}$, a value very similar to that obtained initially. The peak occurred in the first collection period, and the acid levels remained enhanced above baseline for 30 minutes, producing a cumulative increase in acidity of 0.69μmol hr^{-1}. This again was comparable with the total increase in acidity obtained following the initial intravenous injection of G-CA.

Finally, in order to ensure that the solution consisting of the components of the conjugate was able to elicit a change in gastric acid secretion, we decided to intravenously inject 15μg kg^{-1} of the same stock solution already infused ileally

(fourth arrow). This produced a peak increase of 0.54μmol $15min^{-1}$ in the first 15 minutes following infusion, and a cumulative increase in acidity of 1.16μmol hr^{-1}. The elevated response appeared to last for 45 minutes before returning to baseline. We were therefore satisfied that the stock solution was biologically active and, furthermore the presence of glycocholic acid did not exert any inhibitory action on the effectiveness of tetragastrin in inducing an increase in gastric acid secretion.

This experiment was carried out on five rats, and the results produced were very much in agreement (Table 3-8). Intravenous infusion of G-CA (15μg kg^{-1}) resulted in a significant mean peak enhancement of acidity of 0.47 ± 0.14μmol $15min^{-1}$ (P=0.002), occurring in either the first (n=2) or second (n=3) collection period. The total mean increase in acidity above baseline was found to be 0.84 ± 0.35μmol hr^{-1} (P=0.007), with enhanced acidity lasting for between 30 and 45 minutes.

Table 3-8: Peak and cumulative (cum.) increases in gastric acid secretion in response to ileally-administered tetragastrin/glycocholic acid (G4/Gly) (600μg kg^{-1} total in 3:2 ratio) over 180 minute period, intravenous G-CA (15μg kg^{-1}) as the first and third procedure, and intravenous tetragastrin/glycocholic acid (15μg kg^{-1} in 3:2 ratio) as the final procedure.

Rat	Initial I.V. G-CA		Ileal G4/Gly	Second I.V. G-CA		I.V. G4/Gly	
	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})	Cumulative increase (μmol $180min^{-1}$)	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})
1	0.43	1.43*	-0.04	0.67	1.99*	-	-
2	0.30	0.53	-0.47	0.28	0.28	0.43	0.88
3	0.54	0.75	0.05	0.46	0.84	0.20	0.47
4	0.42	0.64	-0.18	0.48	0.69	0.54	1.16
5	0.66	0.84	0.05	0.54	0.71	0.73	1.84
Mean	0.47	0.84	-0.12 ± 0.22	0.49	0.90	0.48	1.09
± S.D.	± 0.14	± 0.35		± 0.14	± 0.64	± 0.22	± 0.58
P-value	0.002	0.007	0.28	0.002	0.035	0.023	0.033

P-values denote statistical significance for comparison with baseline.

* *denotes establishment of new baseline which was elevated compared with that at start of experiment.*

\- *denotes procedure not carried out.*

All five experiments demonstrated very little change in acidity in response to ileal infusion of 600μg kg^{-1} tetragastrin/glycocholic acid (3:2 ratio). A mean drop in acidity of 0.12 ± 0.22μmol 180min^{-1} occurred, which was not significantly different from baseline levels (P=0.28). Intravenous injection of G-CA (15μg kg^{-1}) for a second time resulted in similar increases in gastric acidity as the first. A significant mean peak increase of 0.49 ± 0.14μmol 15min^{-1} (P=0.002) was found to have been evoked, occurring in the first (n=4) or second collection period (n=1). The total increase in acidity above baseline was discovered to be significant and of a value of 0.90 ± 0.64μmol hr^{-1} (P=0.035), and the response was found to last for between 30 and 45 minutes, as was noted previously.

To confirm the biological activity of tetragastrin/glycocholic acid (3:2 ratio), we administered 15μg kg^{-1} of the stock solution intravenously, and discovered that a mean peak increase in acidity of 0.48 ± 0.22μmol 15min^{-1} was evoked (P=0.023), typically in the first or second collection period (n=4 and n=1, respectively), which was statistically significant. The total mean increase in acidity above control levels was calculated as being 1.09 ± 0.58μmol hr^{-1} (P=0.033). Such a response to intravenous administration of tetragastrin/ glycocholic acid (3:2 ratio) confirmed that the preparations were viable in the latter stages of the experimental procedures. Furthermore, ileally-infused tetragastrin and glycocholic acid (3:2 ratio) was an "active" preparation, as confirmed by the responsiveness to intravenous injection, and thus, tetragastrin was fully able to exert its biological activity without being compromised by the presence of glycocholic acid. It would appear, therefore, that tetragastrin requires to be chemically coupled to cholic acid rather than simply be in its presence in order to evoke enhanced gastric acid levels. In addition, these experiments enable us to rule out the unlikely possibility that intravenously administered G-CA, *per se*, increases the permeability of the ileal wall, thereby allowing the transmucosal passage of G-CA and tetragastrin (as seen in the previous set of experiments), resulting in elevated gastric acid levels.

3.1.4 Gastric acid secretion in response to jejunally-administered tetragastrin and tetragastrin-cholic acid conjugate (G-CA)

The mechanism by which ileally-infused G-CA exerted its stimulatory effect on gastric acid secretion was puzzling, since our histological studies signified no apparent structural changes to the intestinal wall (Section 3.3), and yet subsequent ileal administration of tetragastrin resulted in a prominent elevation of basal gastric acid levels, not seen in our previous control experiments. To this end, we felt it necessary to carry out a series of experiments which would bring us closer to determining the mechanisms behind ileal G-CA-induced gastric acid secretion. The effect of jejunally-administered G-CA was investigated, our reasoning being that if structural damage to the ileal wall were occurring, resulting in the transfer of G-CA into the circulation, similar effects would be expected in the jejunum. However, should the site of action be through the bile salt transporter, which is exclusive to the ileum, similar elevation of gastric acid levels in response to jejunally-infused G-CA would not be expected.

3.1.4(i) *Effect of intra-jejunal infusion of tetragastrin on gastric acid secretion*

A series of control experiments were carried out, whereby we investigated the ability of jejunally-infused tetragastrin to elevate gastric acid levels above baseline (Table 3-9). The experiments followed along the same lines as our previous control experiments, in that we first demonstrated the responsiveness of the preparation by administering an intravenous dose of tetragastrin (15μg kg^{-1}). A mean peak increase in gastric acid secretion of 0.44 ± 0.10μmol $15min^{-1}$ was found to be significantly elevated above baseline (P=0.001; n=5). In every case, the peak response was found to occur in the period of injection, that is, between 0 and 15 minutes following administration. The response remained elevated above baseline for between 15 and 30 minutes, resulting in a mean cumulative increase of 0.54 ± 0.17μmol hr^{-1}, which was found to be significant when compared with baseline (P=0.003). Thus, having confirmed unequivocally the viability of the animals with regard to their responsiveness to intravenously administered tetragastrin, we proceeded with the determination of the effectiveness of intra-jejunal infusion of tetragastrin (3000μg kg^{-1} in 1.0ml) in evoking increased gastric acid secretion. The results of this were unequivocal in that there was no evidence to indicate

stimulation of gastric acid secretion in response to jejunal tetragastrin. Over a period of 180 minutes, gastric acid secretion fell by a mean value of 0.05 ± 0.26 μmol 180min^{-1}. This was deemed to be not significantly different from pre-infusion levels (P=0.71).

Table 3-9: Peak and cumulative (cum.) increases in gastric acid secretion in response to jejunally-administered tetragastrin (G4) (3000μg kg^{-1} in 1.0ml saline) over 180 minute period, and to intravenous tetragastrin (15μg kg^{-1}) as a first and final procedure.

Rat	Initial I.V. G4		Jejunal G4	Second I.V. G4	
	Peak increase (μmol 15min^{-1})	Cum. increase (μmol hr^{-1})	Cumulative increase (μmol 180min^{-1})	Peak increase (μmol 15min^{-1})	Cum. increase (μmol hr^{-1})
1	0.33	0.33	-0.25	0.51	0.57
2	0.41	0.50	0.40	0.48	0.57
3	0.61	0.76	-0.06	0.62	0.79
4	0.45	0.45	-0.18	0.53	0.63
5	0.41	0.67	-0.15	0.44	0.54
Mean ± S.D.	0.44±0.10 (P=0.001)	0.54±0.17 (P=0.003)	-0.05 ± 0.26 (P=0.71)	0.52±0.07 (P=0.001)	0.62±0.10 (P=0.001)

P-values denote statistical significance for comparison with baseline.

Our concerns regarding the viability of the preparation towards the latter stages of experimentation were reassured when, on administration of a second intravenous dose of tetragastrin (15μg kg^{-1}), gastric acid levels were significantly elevated above baseline. A mean peak increase of 0.52 ± 0.07μmol 15min^{-1} (P=0.001) was measured, occurring in the period of injection, as was a mean cumulative increase of 0.62 ± 0. 10μmol hr^{-1} (P=0.001). Elevation of gastric acid levels above control levels was maintained for between 30 and 45 minutes before returning to baseline. A paired t-test confirmed the reproducibility of the response to intravenous tetragastrin, since both the peak elevation and the cumulative elevation of gastric acid levels in response to the first and second injection were found to be not significantly different from each other (P=0.056, and P=0.340, respectively).

An example of the outcome of a typical experiment is illustrated in Figure 3-14. Intravenously administered tetragastrin (15μg kg^{-1}) as the first procedure (first arrow) resulted in an elevation of gastric acid secretion to a peak value of

0.41μmol $15min^{-1}$, in the first 15 minutes after injection. The response remained elevated above baseline for 30 minutes, resulting in a cumulative increase of 0.67μmol hr^{-1}. Intra-jejunal infusion of tetragastrin (3000μg kg^{-1}) (second arrow) was ineffective in evoking an increase in gastric acid levels above baseline; a fall of 0.15μmol was actually recorded over a period of 180 minutes.

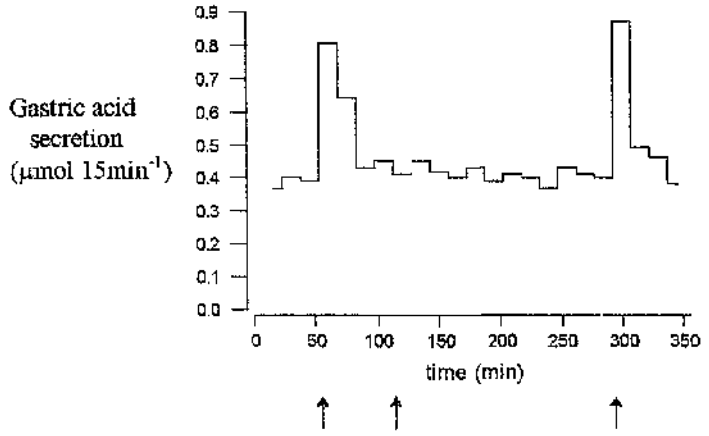


Fig. 3-14: The effect of intravenous injection of tetragastrin (15μg kg^{-1}, first and third arrows) and jejunal infusion of tetragastrin (3000μg kg^{-1} in 1.0ml, second arrow) on gastric acid secretion in the anaesthetised rat (rat 5 in Table 3-9).

Finally, confirmation of the viability of the animal was obtained when, in response to a second intravenous injection of tetragastrin (15μg kg^{-1}) (third arrow), gastric acid levels rose to a peak of 0.44μmol $15min^{-1}$ in the period of injection, and remained elevated for a duration of 45 minutes, resulting in a cumulative increase in gastric acid levels of 0.54μmol hr^{-1}.

3.1.4(ii) *Effect of intra-jejunal infusion of G-CA on gastric acid secretion*

Our previous experiments had demonstrated the inability of jejunally-infused tetragastrin to stimulate increased levels of gastric acid secretion. The effectiveness of jejunally-administered G-CA was then tested. The procedures carried out were similar to those for the previous set of experiments, with the exception that tetragastrin was replaced with G-CA.

The results of a typical experiment, illustrated in Figure 3-15, demonstrated that an initial intravenous injection of G-CA (15μg kg^{-1}) (first arrow) caused an increase in gastric acid levels to a peak value of 0.48 μmol $15min^{-1}$ above baseline in the

period of injection. Because the response lasted for only 15 minutes, the cumulative increase in gastric levels was also 0.48 μmol hr^{-1}.

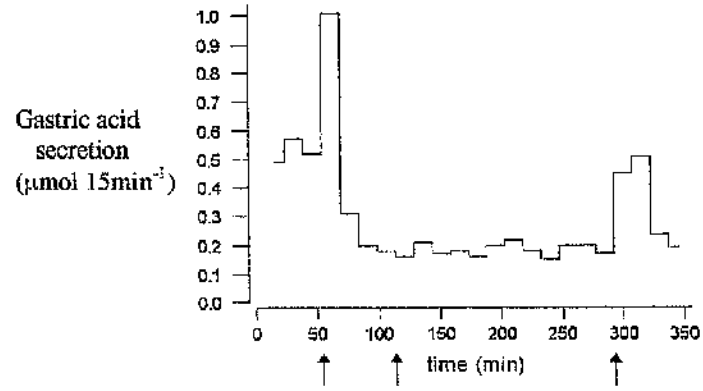


Fig. 3-15: The effect of intravenous injection of G-CA (15μg kg^{-1}, first and third arrows) and jejunal infusion of G-CA (600μg kg^{-1}, second arrow) on gastric acid secretion in the anaesthetised rat (rat 3 in Table 3-10).

Measurement of gastric acid levels over a 180 minute period following jejunally-infused G-CA (600μg kg^{-1} in 1.0ml) (second arrow) provided little evidence to suggest that G-CA had entered the circulation in a biologically active form. Indeed, attenuation of gastric acid levels by 0.08μmol $180min^{-1}$ was recorded. A mean peak increase in gastric acid levels in the period after intravenous injection of G-CA (15μg kg^{-1}) (third arrow) of 0.33 μmol $15min^{-1}$ assured us of the retained responsiveness of the animal in the final stages of experimentation. Sustained elevation of gastric acid levels above baseline for 45 minutes after injection resulted in a cumulative increase of 0.65 μmol hr^{-1}.

Five such experiments were carried out, producing very similar results (Table 3-10). A significant mean peak increase of gastric acid secretion of 0.44 ± 0.13μmol $15min^{-1}$ (P=0.002) was presented in response to initial intravenous infusion of G-CA (15μg kg^{-1}). This generally occurred in either the period of, or the period following injection, and lasted for between 15 and 30 minutes. (In the case of two animals, gastric acid levels did not return to their original value, but remained elevated, and therefore, the duration of response was difficult to define.) This led to a mean cumulative increase in total acidity of 0.58 ± 0.13 μmol hr^{-1}, which was

significant compared to baseline (P=0.001). (The cumulative response of 2.42μmol hr^{-1} presented by one animal was omitted from statistical analysis since it would have biased the results by a disproportionately large amount.)

In each of the five experiments, very little change in gastric acid levels was observed over a 180 minute period following intra-jejunal administration of G-CA (600μg kg^{-1} in 1.0ml). A mean decrease in gastric acid levels of 0.10 ± 0.28 μmol $180min^{-1}$ was found not to be significantly different from baseline (P=0.46).

However, as a final procedure, intravenous infusion of G-CA (15μg kg^{-1}) elicited a significant increase in gastric acid secretion to a mean peak value of 0.42 ± 0.09 μmol $15min^{-1}$ (P=0.001) in the period of injection, and remained elevated for between 15 and 45 minutes, thus resulting in a significant cumulative increase of 0.59 ± 0.16 μmol $hour^{-1}$ (P=0.002). Comparison of the responses to the first and to the second intravenous G-CA injection demonstrated no significant differences between either the mean peak or the mean cumulative increase in gastric acid levels (P=0.60, and P=0.96 respectively). We were, therefore, confident that the lack of response to jejunally-administered G-CA was not due to poor condition of the preparation.

Table 3-10: Peak and cumulative (cum.) increases in gastric acid secretion in response to jejunally-administered tetragastrin-cholic acid conjugate (G-CA) (600μg kg^{-1} in 1.0ml saline) over 180 minute period, and to intravenous G-CA (15μg kg^{-1}) as a first and final procedure.

Rat	Initial I.V. G-CA		Jejunal G-CA	Second I.V. G-CA	
	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})	Cumulative increase (μmol $180min^{-1}$)	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})
1	0.62	2.42*†	0.08	0.55	0.84
2	0.28	0.75*	0.25	0.36	0.52
3	0.48	0.48	-0.08	0.33	0.65
4	0.44	0.44	-0.44	0.39	0.41
5	0.39	0.65	-0.32	0.46	0.51
Mean ± S.D.	0.44±0.13 (P=0.002)	0.58±0.13 (P=0.001)	-0.10 ± 0.28 (P=0.46)	0.42±0.09 (P=0.001)	0.59±0.16 (P=0.002)

P-values denote statistical significance for comparison with baseline.

* *denotes establishment of new baseline which was elevated compared with that at start of experiment.*

† *denotes value omitted from analysis due to disproportionately large value (see text for details).*

3.1.5 Comparison of intra-jejunal and intra-ileal infusion of G-CA on gastric acid secretion

Our final series of experiments was aimed at removing any doubt from our minds that jejunally-infused G-CA was ineffective in evoking elevated gastric acid levels simply as a result of diminished activity of stock solutions. Since earlier experiments had demonstrated the effectiveness of ileally-administered G-CA in eliciting increased levels of gastric acid, we decided to compare the responsiveness of the same rat to intra-jejunal G-CA (600μg kg^{-1} in 1.0ml), followed 180 minutes later with intra-ileal G-CA (600μg kg^{-1} in 1.0ml). This was carried out in five rats, the results of which are described below. Figure 3-16 illustrates the sequence of procedures and the results obtained in a typical experiment.

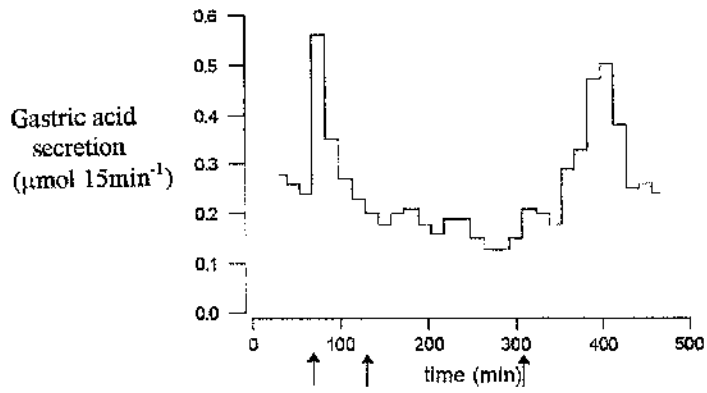


Fig. 3-16: The effect of intravenous injection of G-CA (15μg kg^{-1}, first arrow), jejunal infusion of G-CA (600μg kg^{-1} in 1.0ml, second arrow) and ileal infusion of G-CA (600μg kg^{-1} in 1.0ml, third arrow) on gastric acid secretion in the anaesthetised rat (rat 1 in Table 3-11).

As a matter of course, an initial intravenous injection of G-CA (15μg kg^{-1}) was administered in order to demonstrate the responsiveness of the preparation. A mean peak increase in gastric acid levels of 0.35 ± 0.05 μmol $15min^{-1}$ was presented, and shown to be significant compared to baseline (P=0.001), as was the mean cumulative increase of 0.44 ± 0.10 μmol hr^{-1} (P=0.004) (Table 3-11). (The calculated mean cumulative increase did not take into account the elevated response, presented by one rat, of 1.12μmol hr^{-1}, since this was outwith the normal

distribution of responses.) In all five experiments, the peak response occurred in the period of injection, and gastric acid levels remained elevated above baseline for between 15 and 30 minutes. The results illustrated in Figure 3-16 demonstrate the nature of the response to intravenous G-CA (first arrow), in that a peak increase in gastric acid levels, presented in the period of injection, of 0.30μmol $15min^{-1}$ was observed. Gastric acid levels remained elevated for 30 minutes, totalling a cumulative increase above baseline of 0.39μmol hr^{-1}.

Table 3-11: Peak and cumulative increases in gastric acid secretion in response to I.V. tetragastrin-cholic acid conjugate (G-CA) (15μg kg^{-1}) as a first procedure, subsequently administered jejunal G-CA (600μg kg^{-1} in 1.0ml saline) over 180 minute period, and ileally-administered G-CA (600μg kg^{-1} in 1.0ml saline) as a final procedure.

Rat	Initial I.V. G-CA		Jejunal G-CA	Ileal G-CA
	Peak increase (μmol $15min^{-1}$)	Cumulative increase (μmol hr^{-1})	Cumulative increase (μmol $180min^{-1}$)	Cumulative increase (μmol $180min^{-1}$)
1	0.30	0.39	-0.93	1.41
2	0.36	0.36	-0.57	1.82
3	0.32	1.12*†	-1.09	1.26
4	0.43	0.43	-0.87	1.63
5	0.32	0.59	-0.06	2.02
Mean ± S.D.	0.35 ± 0.05 (P=0.001)	0.44 ± 0.10 (P=0.004)	-0.70 ± 0.41 (P=0.018)	1.63 ± 0.31 (P=0.001)

P-values denote statistical significance for comparison with baseline.

* *denotes establishment of new baseline which was elevated compared with that at start of experiment.*

† *denotes value omitted from analysis due to disproportionately large value (see text for details).*

On return of gastric acid levels to baseline, jejunal infusion of G-CA (600μg kg^{-1} in 1.0ml) was carried out (second arrow), as in the previous series of experiments. Over the following 180 minute period, gastric acid levels were measured and, consistent with earlier results, there was no evidence to suggest any enhancement of gastric acid secretion. In actual fact a significant mean reduction in gastric acid levels of 0.70 ± 0.41μmol $180min^{-1}$ (P=0.018) was observed in 5 rats. The results of the individual experiment (Fig. 3-16) demonstrated a total drop in gastric acid levels of 0.93μmol $180min^{-1}$.

As the final procedure, we introduced G-CA (600μg kg^{-1}) intra-ileally (third arrow) and measured the gastric acid levels that resulted, in order to determine whether the inability of jejunally-administered G-CA was as a consequence of diminished biological activity of the stock solution. The results, in which an increase in gastric acid secretion to a mean peak of 0.42 ± 0.13μmol $15min^{-1}$ above baseline was statistically significant (P=0.002), were convincing. Figure 3-16 illustrates a rise in gastric acid levels to a peak of 0.33μmol $15min^{-1}$ occurring in the 7th collection period; however, the time of occurrence of such a peak in the four other animals was variable, presenting itself in either the 3rd, 4th, 5th or 7th collection period after infusion. Elevation of gastric acid levels above baseline in response to ileally-infused G-CA was maintained for a duration of between 90 and 135 minutes after infusion, resulting in a mean total enhanced gastric acid secretion of 1.63 ± 0.31μmol, which was deemed to be significant (P=0.001). The results of the individual experiment support this finding (Fig. 3-16); gastric acid levels remained elevated for 120 minutes, amounting to a total enhancement of gastric acid levels above baseline of 1.41μmol. We were therefore confident that the ineffectiveness of jejunally-administered G-CA in eliciting increases in gastric acid secretion was due to physiological factors rather than the chemical instability of G-CA.

3.2 Arterial blood pressure

This was recorded to check that the gastrointestinal tract was adequately perfused during the course of what turned out to be extended experiments of up to ten hours duration. Of particular concern was the possibility, in those cases when responses were not evoked, that this might be attributable to poorer perfusion of the internal viscera.

In the initial analysis, the systolic and diastolic blood pressure were measured, and the diastolic pressure was taken to represent the basal tone of the systemic vasculature. The pulse pressure, that is, the difference between systolic and diastolic pressure, was taken to represent a measure of the cardiac contractility. For ease of comparison, the mean arterial blood pressure (MABP) was also calculated.

After completion of surgical procedures and prior to the commencement of the experimental procedures, the arterial blood pressure was typically 135/107mmHg with a MABP of 116mmHg. For all the experimental animals, a MABP (± S.D.) of 117 ± 9mmHg (n=67) was recorded. It was clear that as the experiments progressed, there was a steady decline in arterial blood pressure. Figure 3-17 presents the mean arterial blood pressure and the pulse pressure values obtained from a typical animal during an experiment of approximately 500 min duration (but not including surgical procedures).

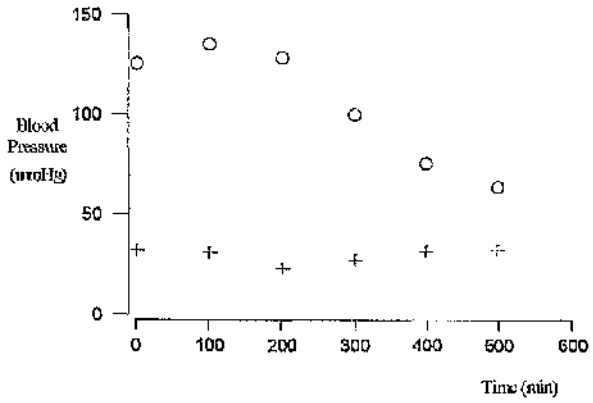


Fig. 3-17: Illustration of the MABP (o) and pulse pressure (+) over time, recorded in the same anaesthetised rat (rat 5 in Table 3-7). Time zero reflects the time after completion of surgical procedures but prior to commencement of experimental procedures.

Over an initial period of 200 min (after completion of surgical procedures), a relatively high MABP was maintained at a value of approximately 130mmHg. A gradual decline then followed over a period of 300 min (between 200 and 500 min after completion of surgical procedures), so that towards the end of the experiment, a MABP of 64mmHg was recorded. This was a typical response observed in all animals. However, balanced against this was the fact that, over the same duration, the pulse pressure changed very little, an observation again seen in all animals. The typical example described in Figure 3-17 shows the pulse pressure of the rat remaining at a fairly steady value of approximately 30mmHg over a 500 min duration. This suggested that the cardiac contractility was being maintained over this period of time. Further, it was observed from an examination of the blood pressure records that the intravenous or intestinal injection of tetragastrin, G-CA, or a mixture of tetragastrin and glycocholic acid was without a sustained effect on arterial blood pressure. Occasionally there was observed a transient disturbance which was attributed to the physical disturbance caused by the injection.

3.2.1 Comparison of arterial blood pressure in responsive and unresponsive rats to intestinally-administered agents

3.2.1(i) *Intravenously-administered tetragastrin and G-CA*

At the end of each experiment, the responsiveness of the preparation was confirmed by the increase in gastric acid secretion in response to intravenously administered tetragastrin or G-CA, at a level not significantly different from that evoked by the initial injections of tetragastrin or G-CA given at the start of the experiment. Nonetheless, the MABP was considerably lower when the response to the second intravenous injection was being recorded, compared with the MABP during the response to the initial intravenous injection of tetragastrin or G-CA. The MABP values are presented in Tables 3-12 and 3-13. The comparable increases in gastric acid secretion in response to the second I.V. injection of either tetragastrin or G-CA, as appropriate, thus reflect the satisfactory metabolic status of the parietal cells which have high metabolic demands, though it does not necessarily indicate the metabolic state of the small intestine.

Table 3-12: Mean arterial blood pressure expressed as the mean value (± S.D.) in each series of experiments investigating the effect of intravenously and ileally administered tetragastrin on gastric acid secretion.

Expt.	Mean arterial blood pressure (mmHg ± S.D.)									
	Time after initial tetragastrin (I.V.)			Time after ileal tetragastrin				Time after second tetragastrin (I.V.)		
	0 min	60 min	**acid**	0 min	90 min	180 min	**acid**	0 min	60 min	**acid**
1	121 ± 12	117 ± 15	**yes**	75 ± 15	-	61 ± 18	**yes**	66 ± 18	38 ± 12	**yes**
2	102 ± 18	94 ± 15	**yes**	88 ± 21	76 ± 14	-	**no**	79 ± 13	62 ± 17	**yes**
3	102 ± 20	99 ± 12	**yes**	82 ± 26	81 ± 20	-	**no**	83 ± 18	68 ± 19	**yes**
4	95 ± 21	83 ± 16	**yes**	87 ± 11	-	60 ± 15	**no**	62 ± 16	52 ± 11	**yes**

'acid' signifies whether the employed procedure resulted in increased levels of gastric acid secretion.

Experiment 1: Ileal tetragastrin (3000μg kg^{-1}) administered subsequent to ileal G-CA, over 180min period (n=7) (Section 3.1.3(iii)).
Experiment 2: Ileal tetragastrin (600μg kg^{-1}) over 90min period (n=11) (Section 3.1.1(ii)).
Experiment 3: Ileal tetragastrin (2500μg kg^{-1}) over 90min period (n=4) (Section 3.1.1(iii)).
Experiment 4: Ileal tetragastrin (2500μg kg^{-1}) over 180min period (n=6) (Section 3.1.1(iv)).

Table 3-13: Mean arterial blood pressure expressed as the mean value (± S.D.) in each series of experiments investigating the effectiveness of intestinal and intravenous G-CA and tetragastrin (G4) in evoking increased gastric acid secretion.

Expt.	Mean Arterial Blood Pressure (mmHg ± S.D.)											
	Time after initial G-CA or tetragastrin$^{\times}$ (I.V.)			Time after jejunal G-CA or tetragastrin$^{\times}$			Time after ileal G-CA			Time after second G-CA or tetragastrin$^{\times}$ (I.V.)		
	0 min	60 min	**acid**	0 min	180 min	**acid**	0 min	180 min	**acid**	0 min	60 min	**acid**
1*	123 ± 13	103 ± 11	**yes**	-	-	-	110 ± 17	93 ± 17	**yes**	89 ± 9	79 ± 20	**yes**
2	121 ± 12	117 ± 15	**yes**	-	-	-	105 ± 25	79 ± 9	**yes**	60 ± 18	38 ± 12	**yes**
3$^{\times}$	114 ± 23	103 ± 23	**yes**	96 ± 15	72 ± 11	**no**	-	-	-	66 ± 7	53 ± 10	**yes**
4	111 ± 9	101 ± 13	**yes**	96 ± 14	73 ± 22	**no**	-	-	-	66 ± 16	57 ± 17	**yes**
5	94 ± 11	94 ± 17	**yes**	91 ± 14	76 ± 6	**no**	71 ± 10	34 ± 21	**yes**	-	-	-

'acid' signifies whether the employed procedure resulted in increased levels of gastric acid secretion.
'' the results of the two rats in which ileal G-CA did not elicit a measurable increase in gastric acid secretion are omitted from this table.*
'×' signifies the administration of tetragastrin, as opposed to G-CA.

Experiment 1: Ileal G-CA (600μg kg^{-1}) administered over 180min period (n=5) (Section 3.1.3(ii)).
Experiment 2: Ileal G-CA (600μg kg^{-1}), administered prior to ileal tetragastrin, over 180min period (n=7) (Section 3.1.3(iii)).
Experiment 3: Jejunal tetragastrin (3000μg kg^{-1}) over 180min period (n=5) (Section 3.1.4(i)).
Experiment 4: Jejunal G-CA (600μg kg^{-1}) over 180min period (n=5) (Section 3.1.4(ii)).
Experiment 5: Jejunal G-CA (600μg kg^{-1}), administered prior to ileal G-CA, over 180min period (n=5) (Section 3.1.5).

3.2.1(ii) *Intra-ileal tetragastrin*

There is the possibility that a compromised metabolic status of the small intestine may have been responsible for the failure of those animals, in which tetragastrin or G-CA was administered intestinally, to demonstrate an increase in total gastric acidity. Therefore, a comparison of the mean arterial blood pressure was made between animals in which no response was obtained, and those animals in which the same (or similar) procedure had been successful in eliciting gastric acid secretion, in order to provide a means of assessment of the relative metabolic status of the small intestine.

When ileally-infused tetragastrin (3000μg kg^{-1}) was undertaken after prior infusion of G-CA (600μg kg^{-1}), it elicited a marked increase in gastric acid secretion (Fig. 3-11). In this case, at the time of intra-ileal administration, a typical blood pressure of 77/58mmHg (MABP = 64mmHg) was recorded. After a further 180 minutes had elapsed, blood pressure had fallen to 70/50mmHg (MABP = 57mmHg) (Fig. 3-18A). Thus, although mean arterial blood pressure for the period of intra-ileal infusion had declined to approximately one-half of the value at the start of the experimental procedures (Section 3.2), the preparation was still responsive to the intra-ileal stimulus.

By contrast, intra-ileal infusion of tetragastrin (600 and 2500μg kg^{-1}) over a 90 min or 180 min period, without the conditioning infusion of G-CA, did not elicit increased gastric acid secretion (Figs. 3-2, 3-3 and 3-4). In these cases, the rats showed much higher systolic and diastolic blood pressures. For example, in one instance, a typical blood pressure at the time ileal infusion of tetragastrin (600μg kg^{-1}) was 112/81mmHg, while at 90 min a value of 94/62mmHg was recorded (Fig. 3-18B). A second animal demonstrated a typical blood pressure of 119/88mmHg at the time of ileal administration of tetragastrin (2500μg kg^{-1}), which gradually fell to 100/72mmHg over a period of 90 min (Fig. 3-18C). The blood pressure in a third animal at the time of administration of tetragastrin (2500μg kg^{-1}) was 107/74mmHg while, at 180 min, this had fallen to 104/65mmHg (Fig. 3-18D). Therefore, an absence of a response to ileally-infused tetragastrin was associated with a notably higher blood pressure than in those animals in which the ileal tetragastrin did elicit a response. This was also the case for the recorded pulse pressure which ranged from 28 to 39mmHg in unresponsive animals, compared with 19 to 20mmHg in the responsive animals. It

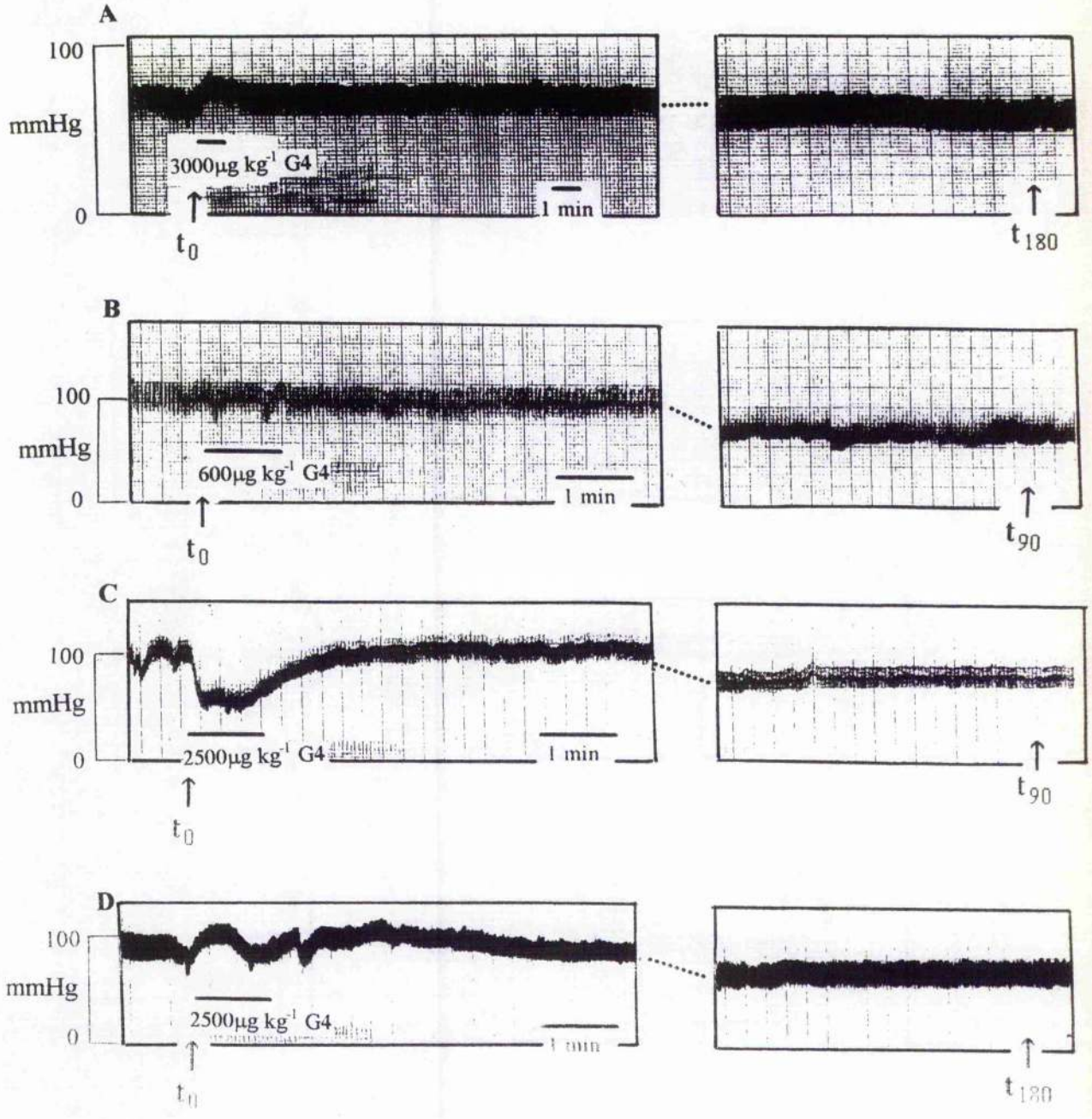


Fig. 3-18: Blood pressure records in which (A), ileal tetragastrin (G4) (3000µg kg^{-1}) evoked increased levels of gastric acid secretion over a period of 180 min (rat 7 in Table 3-7), and (B, C and D), in which ileal tetragastrin (G4) (600 or 2500µg kg^{-1}) evoked no increase in gastric acid levels over 90 or 180 min period (rat 7 in Table 3-2; rat 4 in Table 3-3; rat 1 in Table 3-4, respectively). (Dotted line represents central segment of record which has been omitted. t_0 represents time zero of ileal administration of tetragastrin, and t_{90} and t_{180} indicate 90 and 180 min after administration, respectively.)

would, thus, appear there is little evidence to suggest that the absence of a response to ileally-infused tetragastrin arose from impaired vascular perfusion. Table 3-12 presents the mean blood pressure measurements of the animals employed in investigation of the response to ileal infusion of tetragastrin, and confirms that an absence of response is associated with a higher blood pressure than animals in which an increase in gastric acid secretion was observed.

Further evidence that blood pressure *per se* was not a limiting factor is provided by the recorded values at the time of the initial and second intravenous injections of tetragastrin, as shown in Table 3-12, and the initial and second intravenous injections of G-CA (or tetragastrin), as shown in Table 3-13. These illustrate that a lowered blood pressure at the time of the second I.V. injection is not associated with a reduced gastric acid secretory response.

3.2.1(iii) *Intra-ileal infusion of tetragastrin and glycocholic acid*

The absence of response to ileally-infused tetragastrin and glycocholic acid (in ratio of 3:2; 600μg kg^{-1} in total) (Fig. 3-13) was also unlikely to be attributable to the reduced perfusion of the animal, since the recorded blood pressure in a typical experiment was 122/100mmHg at the time of administration, falling only to 114/86mmHg, 180 min later, which suggested that blood flow to the small intestine was not compromised (Fig. 3-19). Indeed, analysis of the recorded blood pressure obtained from all six rats

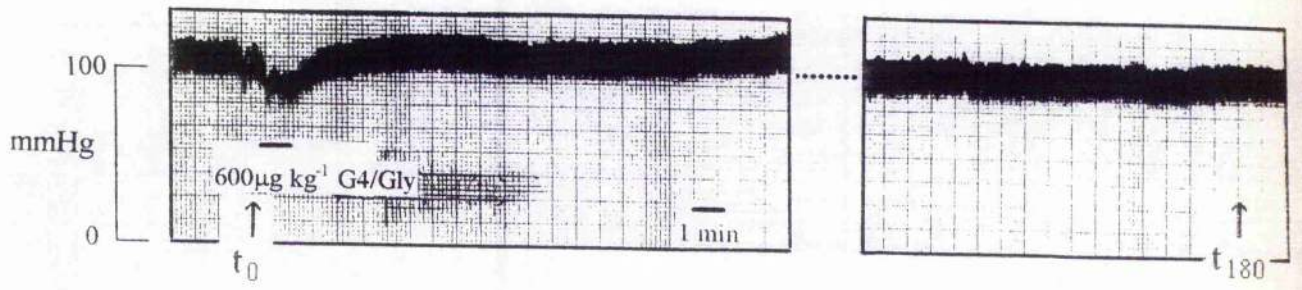


Fig. 3-19: Arterial blood pressure record of rat (rat 2 in Table 3-8) in which ileal infusion of tetragastrin (G4) and glycocholic acid (Gly) (in 3:2 ratio, total dose of 600μg kg^{-1}) did not evoke increased levels of gastric acid secretion over a period of 180 min. (Dotted line represents central segment of record which has been omitted. t_0 represents time zero of administration and t_{180} indicates 180 min after administration.)

used in such procedures demonstrated a mean arterial blood pressure (± S.D.) of 110 ± 5mmHg at the time of ileal administration, which fell gradually to 96 ± 8mmHg after 180 min. Reference to Table 3-12 indicates that gastric acid secretion may be observed at a very much lower blood pressure, and we can thus assume that in this particular experiment, the absence of response to the individual components of G-CA was not attributable to restricted blood flow to the intestine.

3.2.1(iv) *Intra-jejunal infusion of tetragastrin*

Tetragastrin, infused jejunally, did not elicit any increases in gastric acid levels (Fig. 3-14), even though measured blood pressure values suggested that the perfusion state of the small intestine was not compromised. In a typical experiment, on intra-ileal administration of tetragastrin (3000μg kg^{-1}), blood pressure was recorded as 114/81mmHg, giving a MABP of 92mmHg, and a pulse pressure of 33mmHg (Fig. 3-20). After a duration of 180 min, the blood pressure, and hence MABP, had fallen to 103/64mmHg and 77mmHg, respectively, while an increase in pulse pressure to 39mmHg was observed. These are considerably higher values than recorded in the animals in which ileal tetragastrin, injected after G-CA, evoked increased levels of total gastric acidity (e.g. Fig. 3-18A), yet there was no evidence of an increase in gastric acid secretion. The higher level of blood pressure and pulse pressure provided us with assurance that the absence of a response in this experiment was not as a consequence of compromised perfusion of the jejunum.

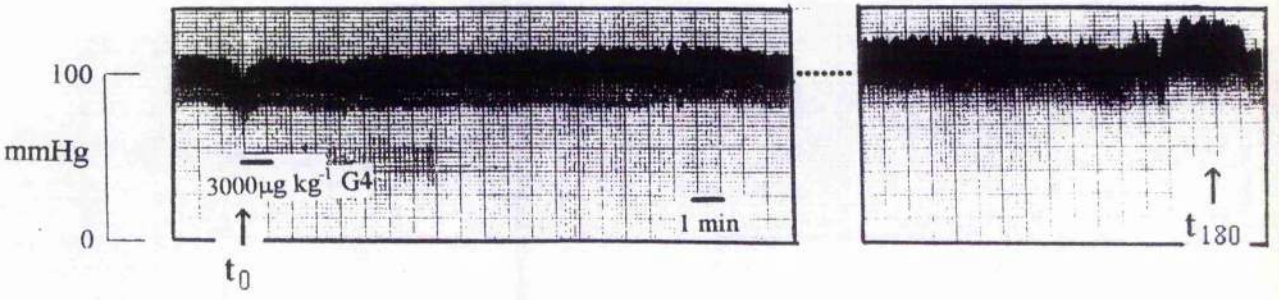


Fig. 3-20: Arterial blood pressure record of rat (rat 3 in Table 3-9) in which jejunal tetragastrin (G4) (3000μg kg^{-1}) did not evoke increased levels of gastric acid secretion over a period of 180 min. (Dotted line represents central segment of record which has been omitted. t_0 represents ileal administration of tetragastrin, and t_{180} indicates 180 min after administration.)

3.2.1(v) *Intra-jejunal infusion of G-CA*

As with jejunally-infused tetragastrin, intra-jejunal G-CA (600μg kg^{-1}) did not elicit any increase in the level of gastric acid secretion (Figs. 3-15 and 3-16), while recorded blood pressure values indicated that this was not attributable to impaired vascular perfusion in the rat. For example, in a typical experiment, a blood pressure of 100/70mmHg (MABP = 80mmHg), recorded at the time of administration of jejunal G-CA, fell to 96/57mmHg (MABP = 70mmHg) after 180 min (Fig. 3-21A). Over this duration, pulse pressure actually increased from 30 to 39mmHg.

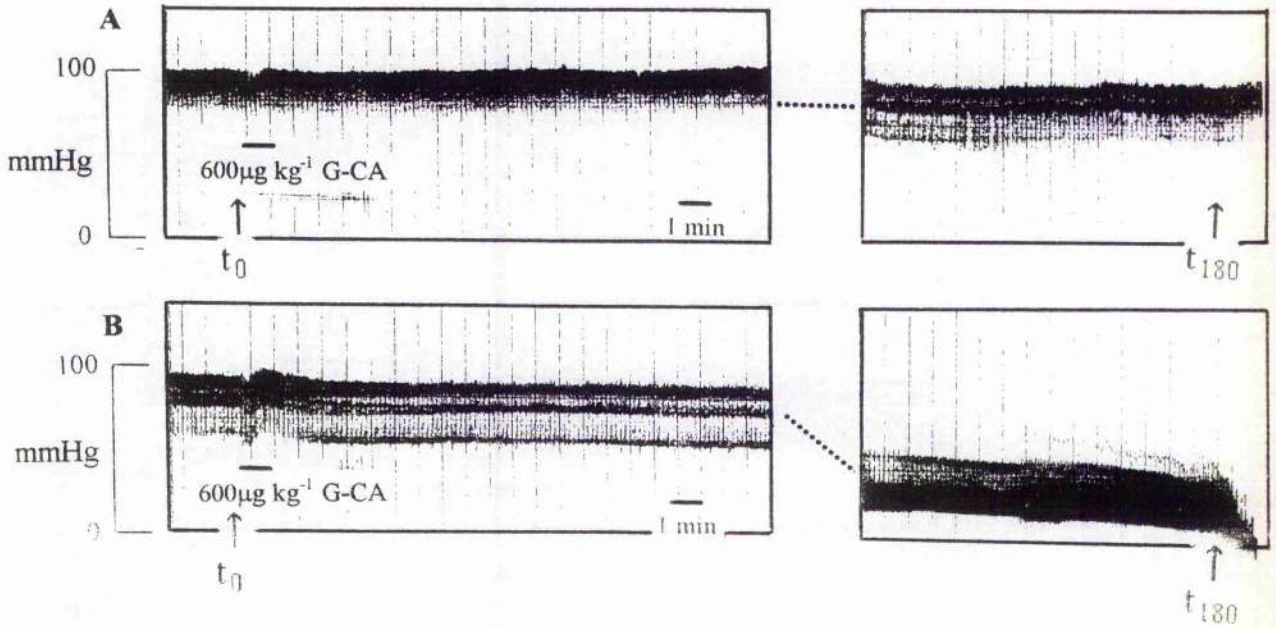


Fig. 3-21: Arterial blood pressure records of the same rat (rat 3 in Table 3-11) in which A, jejunal G-CA (600μg kg^{-1}) did not evoke increased levels of gastric acid secretion over a period of 180 min, and B, ileal G-CA (600μg kg^{-1}) did evoke an increase in gastric acid levels over a 180 min period. Record also shows point of demise of animal at t_{180}. (Dotted line represents central segment of record which has been omitted. t_0 represents time zero of administration of G-CA and t_{180} indicates 180 min after administration.)

By contrast, when intra-jejunal infusion was followed by intra-ileal infusion of G-CA, the blood pressure was considerably lower, and yet an increased level of secretion of gastric acid was elicited (Fig. 3-16). At the time of ileal administration of G-CA, a typical blood pressure in the same rat as above was 91/54mmHg (MABP = 66mmHg), while after a further 180 min this had diminished to 47/16mmHg (MABP = 26mmHg) (Fig. 3-21B). Even at this low value of blood pressure, an increase in

gastric acid secretion was, nevertheless, evoked in response to ileally-administered G-CA.

The mean arterial blood pressure of all experimental animals, expressed as the mean ± S.D., in each of the experiments designed to investigate the stimulatory effect of ileally- and jejunally-infused G-CA (and jejunally-infused tetragastrin) are tabulated (Table 3-13). These results provide unequivocal evidence that even at low blood pressures, gastric acid secretion can be evoked.

3.2.1(vi) *Summary of intestinal infusion results*

From Table 3-14, the mean arterial blood pressures of the rats in which ileal or jejunal infusion of tetragastrin or G-CA did not result in increased gastric acid secretion (experiments 1-7) were indistinguishable from those of the animals in which responses were evoked (experiments 8-11). We were thus confident that the absence of response in the former cases were not attributable to poorer perfusion of the internal viscera.

Table 3-14: Mean arterial blood pressure, expressed as the mean (± S.D.), of animals in which intestinal infusion of either tetragastrin (G4), G-CA or tetragastrin/glycocholic acid was carried out. The response of the preparation to the administered agents is also presented.

Experiment	Mean arterial blood pressure (mmHg) (± S.D)				
	t_0	t_{90}	t_{180}	acid	n
1 (Section 3.1.1(ii))	88 ± 21	76 ± 14	-	**no**	11
2 (Section 3.1.1(iii))	82 ± 26	81 ± 20	-	**no**	4
3 (Section 3.1.1(iv))	87 ± 11	-	60 ± 15	**no**	6
4 (Section 3.1.3(iv))	110 ± 15	-	93 ± 17	**no**	5
5 (Section 3.1.4(i))	96 ± 15	-	72 ± 11	**no**	5
6 (Section 3.1.4(ii))	96 ± 14	-	73 ± 22	**no**	5
7 (Section 3.1.5)	91 ± 14	-	76 ± 6	**no**	5
8 (Section 3.1.3(ii))	110 ± 17	-	93 ± 17	**yes**	5*
9 (Section 3.1.3(iii))	105 ± 25	-	79 ± 9	**yes**	7
10 (Section 3.1.3(iii))	75 ± 15	-	61 ± 18	**yes**	7
11 (Section 3.1.5)	71 ± 10	-	34 ± 21	**yes**	5

Experiments 1, ileal tetragastrin (600μg kg^{-1}); and 2, ileal tetragastrin (2500μg kg^{-1}); both over 90 min period. Experiments 3, ileal tetrgastrin (2500μg kg^{-1}); 4, ileal tetrgastrin and glycocholic acid (in 3:2 ratio, 600μg kg^{-1} total dose); 5, jejunal tetragastrin (3000μg kg^{-1}); 6, jejunal G-CA (600μg kg^{-1}); 7, jejunal G-CA (600μg kg^{-1}) prior to ileal G-CA; 8, ileal G-CA (600μg kg^{-1}); 9, ileal G-CA (600μg kg^{-1}) prior to ileal tetragastrin; 10, ileal tetragastrin (3000μg kg^{-1}) subsequent to ileal G-CA; and 11, ileal G-CA (600μg kg^{-1}) subsequent to jejunal G-CA; all over 180 min period.

t_0, time zero of administration; t_{90}, 90 min after administration; t_{180}, 180 min after administration; 'acid' signifies whether the employed procedure resulted in increased levels of gastric acid secretion; n, number of rats; '', omission of results from two rats in which measurable increases in gastric acid levels were not recorded.*

3.3 Histological analysis

Figures 3-22A and B illustrate two representative samples of light microscopic sections of ileal mucosa, removed from a rat in which no instillation of G-CA had occurred. These may be compared with the sections of ileal mucosa excised from a rat in which instillation of G-CA (600μg kg^{-1}) was performed (Figs. 3-22C and D). At this level of resolution, the integrity of the epithelial wall was maintained with no visible damage in both cases. There is no evidence of any major lesions to the ileal mucosa through which enhanced absorption of G-CA may have occurred. In addition, the presence of mucus-releasing goblet cells in both the perfused and unperfused tissue is apparent (Figs. 3-22B and D), as is a well-defined brush-border (Figs. 3-22A and C)

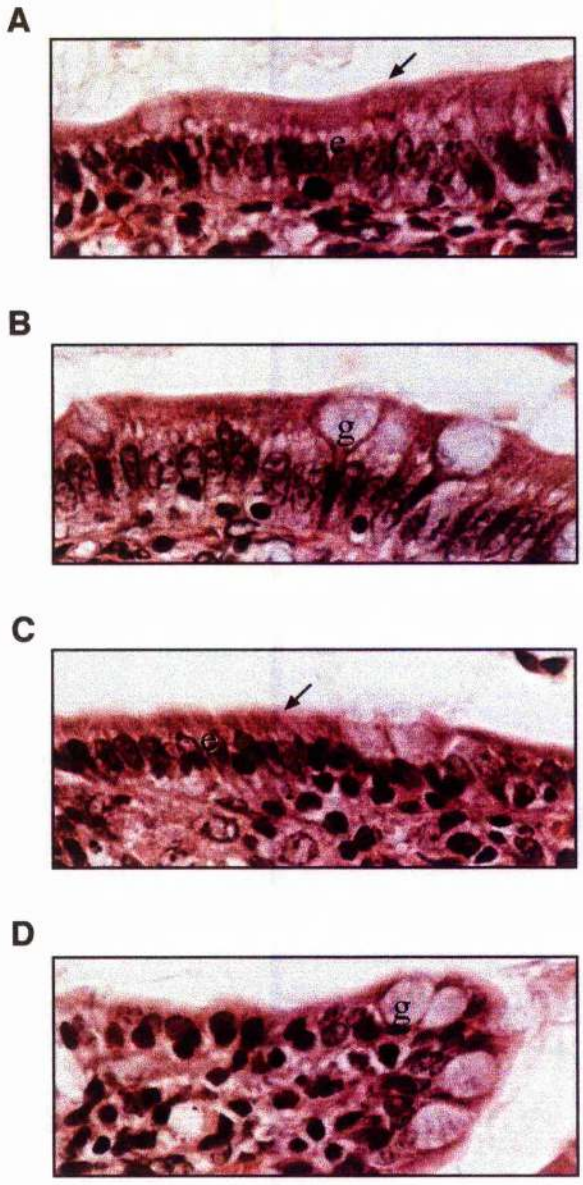


Fig. 3-22: Light microscopic sections of epithelium of ileum, with lumen uppermost, of two rats. A and B, non-perfused ileum; C and D, ileum perfused with 600μg kg^{-1} G-CA. Sections show enterocytes (e), goblet cells (g) and brush border (→). Sections stained with H&E, x700.

DISCUSSION

4.1 Summary of results

The present study has demonstrated a number of unequivocal results. First, intra-intestinal instillation of tetragastrin was shown not to result in a measurable increase in gastric acid secretion, suggesting that the tetrapeptide was not absorbed across the intestinal wall in any measurable amount, if indeed at all. The biological activity of the synthesised tetragastrin-cholic acid conjugate (G-CA) was confirmed when intravenous injection (15μg kg^{-1}) evoked measurable increases in gastric acid output. Furthermore, in contrast with tetragastrin, intra-ileal administration of G-CA (600μg kg^{-1}) caused a marked increase in gastric acid secretion from which it was inferred that it had been transported across the ileal mucosa, while retaining considerable biological activity. The individual components of G-CA, that is, tetragastrin and glycocholic acid (cholic acid was insoluble in isotonic saline and, thus, could not be used) (in the ratio of 3:2; total dose of 600μg kg^{-1}) were shown not to be effective since there was no evidence of an increased level of gastric acid secretion following intra-ileal instillation of these compounds. The transmucosal movement of G-CA appeared to be specific to the ileum, as there was no evidence of transport across the intestinal mucosa following intra-luminal instillation in the jejunum.

4.2 Biological activity of G-CA and tetragastrin

The biological activity of synthetic tetragastrin-cholic acid conjugate (G-CA) was confirmed when intravenous injection (15μg kg^{-1}) evoked an increase in gastric acid secretion from the stomach of the rat preparation. Very good consistency was demonstrated between the responses to the first and second intravenous injections of G-CA within a particular experiment. Between the different sets of experiments, broadly similar results for the mean peak increase in gastric acid output were measured, although there was some variation in the mean cumulative responses (Table 4-1).

With respect to tetragastrin, there was some potentiation of the peak and cumulative responses to the second intravenous injection of tetragastrin (minimum effective doses of between 12.5 and 60μg kg^{-1}) in experiments 1 and 2, whereas experiments 3, 4 and

5 showed no evidence of potentiation for both the peak and cumulative increases in gastric acid secretion following intravenous injection of tetragastrin as a first and final procedure (Table 4-2).

Table 4-1: Mean peak and cumulative (cum.) increases in gastric acid secretion in response to I.V. tetragastrin-cholic acid conjugate (G-CA) (15μg kg^{-1}) as a first and final procedure, as carried out in 6 sets of experiments.

Experiment (showing section of results)	Initial I.V. G-CA		Second I.V. G-CA		n
	Mean peak increase (μmol $15min^{-1}$ ± S.D.)	Mean cum. increase (μmol hr^{-1} ± S.D.)	Mean peak increase (μmol $15min^{-1}$ ± S.D.)	Mean cum. increase (μmol hr^{-1} ± S.D.)	
1 *(3.1.2)*	0.49 ± 0.13	1.10 ± 0.73	0.65 ± 0.28	1.46 ± 0.84	5
2 *(3.1.3(ii))*	0.64 ± 0.26	1.31 ± 0.57	0.72 ± 0.26	1.43 ± 0.62	5
3 *(3.1.3(iii))*	0.57 ± 0.24	1.09 ± 0.52	0.70 ± 0.42	1.15 ± 0.59	7
4 *(3.1.3(iv))*	0.47 ± 0.14	0.84 ± 0.35	0.49 ± 0.14	0.90 ± 0.64	5
5 *(3.1.4(ii))*	0.44 ± 0.13	0.58 ± 0.13	0.42 ± 0.09	0.59 ± 0.16	5
6 *(3.1.5)*	0.35 ± 0.05	0.44 ± 0.10	n/a	n/a	5
Weighted mean ± S.D.	0.50 ± 0.18	0.91 ± 0.47	0.60 ± 0.28	1.11 ± 0.61	32 (1st) 27 (2nd)

All values were statistically significant when compared with zero baseline ($P<0.05$); n, number of animals.
Experimental sections from which results in Table were taken were: 1, intravenous G-CA followed by second intravenous G-CA; 2, ileal G-CA (600μg kg^{-1}) over 180min period; 3, ileal G-CA (600μg kg^{-1}) prior to ileal tetragastrin, over180min period; 4, ileal tetragastrin and glycocholic acid (ratio of 3:2, total dose of 600μg kg^{-1}), over 180min period; 5, jejunal G-CA (600μg kg^{-1}) over 180min period; 6, jejunal G-CA (600μg kg^{-1}) prior to ileal G-CA, over 180 min period.

Reference to the weighted mean peak and cumulative increases in gastric acid, presented in Tables 4-1 and 4-2, in response to the first intravenous injection of G-CA or tetragastrin, as seen in a total of 32 and 31 rats, respectively, demonstrate a general similarity in magnitude. Comparable responses are also shown by the weighted mean peak responses to intravenous G-CA (n=27) and tetragastrin (n=31), as a final procedure, while the weighted mean of the cumulative increase in gastric acid secretion was approximately 47% higher in the rats in which tetragastrin was

administered intravenously, compared with those rats in which G-CA (I.V.) was administered as a final procedure.

Table 4-2: Mean peak and cumulative (cum.) increases in gastric acid secretion in response to intravenous tetragastrin (G4) (between 12.5 and 60μg kg^{-1}) as a first and final procedure, as carried out in 5 sets of experiments.

Experiment (showing section of results)	Initial I.V. G4		Second I.V. G4		n
	Mean peak increase (μmol $15min^{-1}$ ± S.D.)	Mean cum. increase (μmol hr^{-1} ± S.D.)	Mean peak increase (μmol $15min^{-1}$ ± S.D.)	Mean cum. increase (μmol hr^{-1} ± S.D.)	
1 *(3.1.1(i))*	0.53 ± 0.25	1.38 ± 0.84	0.90 ± 0.30	2.25 ± 0.92	5
2 *(3.1.1(ii))*	0.58 ± 0.33	1.27 ± 0.83	0.81 ± 0.53	2.43 ± 1.86	11
3 *(3.1.1(iii))*	0.47 ± 0.16	1.15 ± 0.36	0.36 ± 0.06	0.64 ± 0.29	4
4 *(3.1.1(iv))*	0.42 ± 0.10	0.84 ± 0.27	0.50 ± 0.14	1.20 ± 0.49	6
5 *(3.1.4(i))*	0.44 ± 0.10	0.54 ± 0.17	0.52 ± 0.07	0.62 ± 0.10	5
Weighted mean ± S.D.	0.50 ± 0.06	1.07 ± 0.39	0.66 ± 0.12	1.64 ± 1.42	31

All values were statistically significant when compared with zero baseline ($P<0.05$); n, number of animals.
Experimental sections from which results in Table were taken were: 1, intravenous tetragastrin followed by second intravenous tetragastrin; 2, ileal tetragastrin (600μg kg^{-1}) over 90min period; 3, ileal tetragastrin (2500μg kg^{-1}) over 90min period; 4, ileal tetragastrin (2500μg kg^{-1}) over 180min period; 5, jejunal tetragastrin (3000μg kg^{-1}) over 180min period.

G-CA is composed of tetragastrin and cholic acid in an approximate ratio of 3:2. Thus, the general similarity observed between the intravenous responses to G-CA (15μg kg^{-1}) and tetragastrin (minimum effective dose, most commonly 15μg kg^{-1}) suggests that the same level of response is recorded to G-CA at two-thirds the effective dose of tetragastrin administered. Had the synthesised conjugate contained appreciable amounts of fragments of the entire G-CA molecule, such as tripeptide-cholic acid or dipeptide-cholic acid, neither would be biologically active since the complete tetrapeptide amide sequence is required for full biological activity (Morley, Tracy and Gregory, 1965), the biological activity would be reduced. This lends assurance that G-CA was of reasonable purity.

4.3 Ileal administration of G-CA

The results of the present study demonstrated unequivocally that instillation of the conjugate into the ileum resulted in the stimulation of gastric HCl secretion (Table 4-3). From this we can infer that G-CA has crossed the ileal wall, entered the systemic circulation and subsequently stimulated the parietal cells. The extent to which levels increased varied (shown by individual mean S.D.values), but for 17 rats, the weighted mean was calculated as 1.84 ± 1.22μmol $180min^{-1}$, a value approximately twice as great as the cumulative response to the intravenous injection. Omitted from these results are the two rats in which ileal G-CA elicited marginal or no measurable increases in gastric acid levels (see Table 3-6).

Table 4-3: Mean cumulative increases in gastric acid secretion in response to ileally-infused tetragastrin-cholic acid conjugate (G-CA) (600μg kg^{-1}), as carried out in 3 sets of experiments.

Experiment (showing section of results)	Ileal G-CA Mean cumulative increase (μmol $180min^{-1}$ ± S.D.)	n
1 *(3.1.3(ii))*	2.80 ± 2.17	5
2 *(3.1.3(iii))*	1.31 ± 0.44	7
3 *(3.1.5)*	1.63 ± 0.31	5
Weighted mean ± S.D.	1.84 ± 1.22	17

All values were statistically significant when compared with zero baseline ($P<0.05$); n, number of animals.
Experiments 1, ileal G-CA (600μg kg^{-1}) over 180min period; 2, ileal G-CA (600μg kg^{-1}) prior to ileal tetragastrin, over 180min period; 3, ileal G-CA (600μg kg^{-1}) subsequent to jejunal G-CA, over 180min period.

It is difficult to state accurately the quantity of G-CA reaching the systemic circulation without carrying out radioimmunoassay of the systemic blood. In any case, comparison of the increased level of gastric HCl secretion elicited in response to intra-ileal G-CA, with the response to intravenous G-CA, is perhaps a more reliable way of estimating the absorption of G-CA into the systemic circulation, simply because radioimmunoassay of levels in the bloodstream may be affected by

hydrolysed fragments of G-CA (Ziv *et al*, 1987). So, radioimmunoassay may not have provided any additional information; indeed a degree of ambiguity may have been introduced.

Intravenous injection of G-CA (15μg kg^{-1}), as a first procedure, evoked a mean cumulative increase in gastric acid levels of 0.91 ± 0.47μmol hr^{-1}, and as a final procedure, caused gastric acid secretion to increase by 1.11 ± 0.61μmol hr^{-1} above baseline (Table 4-1). Comparison of these values with the degree to which ileally-infused G-CA (600μg kg^{-1}) enhanced gastric acid secretion (weighted average of 1.84 ± 1.22μmol 180min^{-1}; Table 4-3) would suggest that, on the basis of simple proportion, just under 5% of the intra-ileally administered dose was absorbed from the ileum. This calculation is complicated by the half-lives of tetragastrin and G-CA. The clearance rate (half life) of G17 from the human circulation is approximately six minutes, whereas that for G34 is about six times longer (Walsh *et al*, 1974; Walsh *et al*, 1976). From this we may deduce that the clearance rate for tetragastrin (G4) would be in the region of six minutes or less. Undoubtedly, a considerable amount of tetragastrin may be "wasted" through clearance by the liver and kidneys. This is consistent with the fact that the minimum effective dose of tetragastrin is of the order 12.5μg kg^{-1} and, thus, the instantaneous distribution of tetragastrin in the plasma of the rat (approximately 10ml) results in a plasma concentration which is seemingly very high. The clearance rate of G-CA is unknown, but will involve factors such as the degree to which G-CA is hydrolysed into free tetragastrin, in addition to how much of the administered G-CA is removed by the hepatocytes which avidly take up bile salts. Since the minimum effective dose of G-CA was 15μg kg^{-1}, giving an effective tetragastrin dose of approximately 10μg kg^{-1}, compared with the commonest minimum effective dose of tetragastrin alone of 15μg kg^{-1}, this would indicate a greater efficacy of G-CA possibly resulting from a longer half life.

The encouraging outcome of this study is perhaps more apparent if we consider the comparative oral bioavailability of other peptides, for example, insulin. In general, bioactivity of orally-administered peptides is very low indeed (see Table 1-2). Co-administration of insulin with protease inhibitors and permeation enhancers is one of the more successful approaches and yet, still less than 1.0% of the administered dose reaches the systemic circulation (Ziv *et al*, 1987).

4.3.1 Comparison with ileal and jejunal administration of tetragastrin

The results for ileal administration of G-CA (600μg kg^{-1}) were in contrast to those for ileal infusion of tetragastrin (2500μg kg^{-1}); measurable increases in gastric acid secretion were not elicited in response to the latter (see Tables 3-2, 3-3 and 3-4). This was the case even though the dose of tetragastrin was up to 4 times greater than the dose of G-CA (and thus, on a weight for weight basis, approximately 6.25 times greater than the dose of tetragastrin contained within G-CA). Additionally, jejunal infusion of tetragastrin (3000μg kg^{-1}) produced no discernible increase in gastric acid secretion (see Table 3-9).

Since the intravenous injection of tetragastrin, as a first procedure, demonstrated its biological activity, and as a final procedure, demonstrated its continued biological activity as well as the viability of the animal preparation (see Tables 3-2, 3-3, 3-4 and 3-9), we were confident that the lack of response to intestinal infusion of tetragastrin (as the second procedure) was as a consequence of absence of any substantial level of transmucosal movement. Furthermore, measurement of the arterial blood pressure of these rats, during the infusion of ileal tetragastrin, indicates that the lack of response was not attributable to reduced vascular perfusion, since an increase in gastric acid in response to ileal G-CA has been demonstrated at considerably lower blood pressures (see Tables 3-12 and 3-13). An absence of response may be contributed to by the degradative activity of the proteolytic enzymes in the gut, in particular the intestinal brush border peptidases (listed in Table 1-1a). These are capable of hydrolysing peptides of up to ten amino acid residues, but have a preference for tripeptides (Matthews and Payne, 1980). The wall of the small intestine may also act as a physical barrier to the absorption of tetragastrin since, before targeting the parietal cells, tetragastrin has to penetrate the mucosal membrane of the intestinal wall in order to enter the systemic circulation. Di- and tripeptide transport systems exist in the intestinal brush border membrane, allowing the intact transport of such molecules (Newey and Smyth, 1960; 1962); however, peptides composed of greater numbers of amino acid residues are not taken up. Uptake by hepatocytes of any intestinally-infused tetragastrin which reaches the mesenteric circulation may also play a role in the absence of a measurable increase in gastric acid secretion (Doherty and Pang, 1997). Chemical modification of tetragastrin by acylation with various fatty acids has

been shown to not only increase its lipophilicity, but also reduce degradation of tetragastrin, thereby enhancing intestinal absorption in the rat (Yodoya, Uemura, Tenma, Fujita, Murakami, Yamamoto and Muranishi, 1994). In addition, hepatic first-pass metabolism of the modified tetragastrin was slightly suppressed in these experiments, suggesting that the stability of such compounds in the systemic circulation would be enhanced, compared with tetragastrin.

At variance with the results of the present study is the outcome of a study, carried out in the stomach-perfused rat preparation, in which tetragastrin, injected into the jejunal or ileal loop, produced a measurable increase in gastric acid secretion (Jennewein *et al*, 1974). Jejunal or ileal administration of tetragastrin (10mg kg^{-1}) was reported to elicit a maximal increase in gastric acid secretion of 62% and 80% of the maximal increase in response to intravenous tetragastrin (30μg kg^{-1}), respectively. Intra-duodenal adminstration of tetragastrin did not evoke any measurable increases in gastric acid secretion, though the authors demonstrated that the exclusion of pancreatic enzymes, which led to enhanced absorption from the duodenum, may have been the factor which accounted for the enhanced absorption recorded from the jejunal and ileal loops.

The differences in results reported in this study, and in our study may arise from the different doses administered, since Jennewein *et al* injected tetragastrin into the intestinal loops at a dose approximately 3 times the maximum dose that we instilled. A further consideration may be the means by which tetragastrin was introduced into the intestinal loops. In the present study, leakage of tetragastrin into the internal viscera was prevented by the insertion of a short length of cannula at the site of intestinal instillation, through which compounds were administered. This was deemed necessary since we had observed in earlier experiments that direct injection of pentagastrin (G5) through the ileal wall resulted in a doubling of gastric acid output above control levels, as a consequence of leakage, visible at the site of injection. One is under the impression that Jennewein and colleagues introduced tetragastrin into the intestinal loops by direct injection through the intestinal wall. It is, thus, conceivable that at least some of the tetragastrin may have escaped, and entered the surrounding capillaries, although the absence of a response after intra-duodenal administration tends to exclude this possibility. Nonetheless, despite the resultant increase in gastric

acid secretion, the authors proposed that no more than approximately 0.04% of the administered dose was absorbed after jejunal or ileal loop infusion.

4.3.1(i) *Degree of ionisation of G-CA and tetragastrin solutions*

The dissimilarity observed between the transport of ileally-infused G-CA and the non-transport of ileally-infused tetragastrin may, theoretically, have occurred as a result of differences in the ionisation states of these compounds, since non-ionised forms are absorbed by passive diffusion on the basis of their oil/water partition coefficient. It is well established that, in general, the unionised form of a weak acid or base is able to penetrate the intestinal wall at a much faster rate than the corresponding ionised acid or base, unless there is a specific transport mechanism in operation. Validation of this principle comes from the outcome of investigative studies using artificial membrane systems (Doluisio and Swintosky, 1964; Samuelov, Donbrow and Friedman, 1979), and gastrointestinal membranes such as the gastric mucosa, and the small and large intestines (Hogben, Schanker, Tocco and Brodie, 1957; Shore, Brodie and Hogben, 1957; Schanker, Shore, Brodie and Hogben, 1957; Schanker, Tocco, Brodie and Hogben, 1958; Schanker, 1959; Hogben, Tocco, Brodie and Schanker, 1959).

However, our calculated estimates of the degree to which tetragastrin and G-CA are ionised (see Appendix) appear to refute this proposal. Since both compounds are fairly complex molecules, these calculations had to be carried out by consideration of the degree to which the major side groups (COOH, NH_2 and NH) of the molecules were ionised. In the case of tetragastrin, ionisation was estimated to be 100%, except for COOH which was calculated to be 14% ionised, while for G-CA, with the exception of the carboxyl (COOH) group which was calculated to be about 57% ionised, the degree of ionisation was calculated to be approaching 100%. We can, therefore, confidently rule out differences in the rate of passive diffusion on the basis of lipid solubility as a means of explaining the differences in ileal transport between G-CA and tetragastrin.

4.3.1 (ii) *Permeability changes elicited by bile salts*

Bile salts are well recognised for their efficacy in enhancing transmucosal permeation and, thus, have been widely used as such in studies investigating the potentiation of intestinal permeation of otherwise poorly absorbed macromolecules (Kidron *et al*,

1982; Ziv *et al*, 1987; Bendayan *et al*, 1990). Unfortunately, considerable mucosal damage effected by some bile salts has been reported (Gaginella *et al*, 1977; Nadai *et al*, 1975; Nakanishi *et al*, 1983). There was, thus, a possibility that G-CA, instilled into the ileum, caused an increase in gastric acid secretion through permeability changes to the intestinal wall, elicited by cholic acid contained within G-CA. The results of the present study, however, appear to refute this possibility, since we have shown that simultaneously-infused tetragastrin and glycocholic acid (we were compelled to use glycocholic acid due to the insolubility of cholic acid in isotonic saline), in a ratio of 3:2, into the ileum did not elicit any measurable increases in gastric acid secretion above baseline (see Table 3-8). This was in contrast to the unequivocal response evoked by intravenous infusion of the same solution (at a dose 40 times less than the ileal dose). This allowed us to conclude a number of points.

First, the intravenously administered solution had a very similar biological activity to tetragastrin and G-CA and, thus, the presence of glycocholic acid does not exert an inhibitory action on the activity of tetragastrin.

Second, instillation of G-CA (in conjugated form), rather than simultaneous infusion of the components of G-CA into the ileum, is a prerequisite for the increase in transmucosal transport of tetragastrin with resultant levels which are sufficient to stimulate increased gastric acid secretion. This, perhaps, lends support to the idea that the ileal bile salt carrier is the means by which this occurs during these experiments.

Finally, in both this series of experiments which investigated the biological activity of simultaneously infused tetragastrin and glycocholic acid into the ileum, and the series of experiments in which the activity of ileal G-CA was investigated, the first experimental procedure carried out in each case was an intravenous injection of G-CA. The outcome of the former experiments in which ileal tetragastrin infused along with glycocholic acid did not elicit a measurable increase in gastric acid secretion provides evidence to suggest that the I.V. injection of G-CA did not increase the permeability of the ileal wall, by some mechanism acting from the blood side thereby allowing G-CA to enter the circulation when administered ileally. This is further corroborated by the experiments in which jejunal infusion of G-CA, subsequent to intravenous G-CA, did not elevate gastric acid levels above baseline (see Tables 3-10

and 3-11), and additionally, by the normal histology of the ileal wall, as determined by light microscopic analysis (Figures 3-22A and B) (see later).

4.4 Site-specificity of response to intestinally-administered G-CA

Of considerable interest was the absence of response to jejunally-infused G-CA (600μg kg^{-1}) in the same rats in which responsiveness to intravenously-infused G-CA (15μg kg^{-1}), as first and final procedures, was demonstrated (see Table 3-10). We were, thus, confident that neither the prepared solution of G-CA had lost its biological activity nor that the animal preparation had become unresponsive to the effects of G-CA. Further reinforcement of this view was provided in a further series of experiments when, subsequent to jejunally-infused G-CA (600μg kg^{-1}), which was without effect, ileally-infused G-CA (600μg kg^{-1}) did cause an increase in gastric acid secretion (Tables 3-11 and 4-3). Such an outcome is indicative of a location-specific mechanism, of which there a number of possibilities which require consideration.

4.4.1 Ileal bile salt active transport system

The rationale behind the present study was to produce a tetragastrin-cholic acid conjugate which would have the structural requirements necessary for recognition by the ileal bile salt active carrier system. Utilisation of such a system was envisaged to effect an improved bioavailability of intestinally-administered tetragastrin. Although transport of bile salts occurs across the entire length of the small intestine by both ionic and non-ionic diffusion (Dietschy, 1973), the active carrier-mediated uptake is specifically localised to the ileum (Lack and Weiner, 1961).

A review of the current literature reveals that for optimal recognition of a bile salt by the transport system, the characteristics of naturally occurring bile salts should be retained as far as is possible. Thus, a negative charge in the side chain attached to position C-17, and at least one hydroxyl group at position C-3, C-7 or C-12 of the steroid nucleus (see Figs. 1-2 and 1-3) are necessary (Lack and Weiner, 1967; Anwer *et al*, 1985; Hardison *et al*, 1991; Kramer *et al*, 1993). In addition, it has been determined that conjugate molecules in which the drug moiety is attached to the C-3 position are recognised like natural bile acids by the hepatic bile acid transport system (Kramer *et al*, 1992; Wess, Kramer, Bartmann *et al*, 1992). Indeed, a comprehensive

study in the rat *in vivo*, carried out by Kramer *et al* (1994), demonstrated the uptake of a conjugate of cholic acid and a synthetic tetrapeptide (labelled with an additional fluorescent group) by the ileal bile salt active carrier system, in which the tetrapeptide was coupled to the C-3 position of the steroid nucleus, and the side chain attached to C-17 was unmodified. However, while Kim and colleagues (1993) had previously shown that renin-inhibitory peptides (RIPs), coupled with cholic acid or taurocholic acid at position C-3, resulted in a high affinity binding with the bile salt transporter in the Caco-2 cell, in the perfused rat ileum, measurable amounts of RIP-cholic acid were not detected in the mesenteric blood. This suggested that, although uptake by the ileal bile salt active carrier system was possible, complete transfer of the conjugate to the circulation was impeded, either in passage through the enterocyte, or efflux from the enterocyte.

In the present study, we were of the view that conjugation of tetragastrin with cholic acid at the site at which glycine or taurine naturally attach (C-24) may provide a feasible alternative to the approach of Kramer and colleagues. The negative charge of cholic acid was retained through the presence of the carboxyl group of aspartate. Indeed, our study has produced considerable evidence to demonstrate the transport of G-CA across the ileal wall, but the non-transport of G-CA across the jejunal wall. Although we have no absolute evidence that the bile salt active transport system is being utilised to produce this response, the fact that we are confident that our compound is of reasonable purity and a response was recorded only when G-CA was instilled into the region of small intestine where the bile salt carrier system is localised, lends support that this is indeed the case.

Over the past ten years, previous studies have adopted a set of criteria contrary to those suggested by Lack and Weiner (1967), Anwer *et al* (1985), Hardison *et al* (1991) and Kramer *et al* (1993). As in the present study, modifications at the C-24 position of the bile acid, rather than at the C-3 position of the steroid nucleus, have been investigated as a possible means of facilitating the uptake of otherwise poorly absorbable macromolecules. In a study by Maeda and Takahashi (1989), *para*-aminobenzoic acid (PABA) was covalently coupled to ursodeoxycholic acid at the C-24 position. Consequently, using the rat everted gut sac technique, PABA-ursodeoxycholic acid was reported to be transported against a concentration gradient via the ileal bile salt active carrier system. A subsequent investigation revealed that

conjugation of L-triiodothyronine (L-T_3) with cholic acid at the C-24 position, so that a negative charge of the C-17 side chain remained, resulted in significant hypocholesterolaemic activity following oral administration of the conjugate, *in vivo*, to lipaemic rats (Stephan, Yurachek, Sharif, Wasvary, Steele and Howes, 1992). However, the rationale behind this study was not to increase intestinal uptake; rather, the authors aimed to produce a thyromimetic with a high affinity for the hepatocytes, whilst reducing cardiac and pituitary-associated side effects.

The former study (Maeda and Takahashi, 1989), along with the present study, demonstrates that through the coupling of an otherwise poorly absorbed drug moiety with the C-24 position of bile acids, transfer across the intestinal wall may be achieved. So, while the retention of the negative charge at the bile acid side chain (position C-17) still remains a prerequisite for the successful transportation of macromolecules via the bile salt carrier system, the view that attachment of these compounds could only be at position C-3 of the steroid nucleus of the bile acid has now been refuted.

4.4.2 Ileal localisation of membranous epithelial cells (M-cells)

Lymphoid tissue in the intestinal mucosa becomes especially prominent in the ileum, resulting in the formation of Peyer's patches from the aggregated lymphoid tissue. Overlying these aggregates are the M cells, which have been shown to be able to take up antigens from the gut lumen, and transport them to the underlying lymphocytes (Owen, 1977).

M cells have been found in a number of mammalian species including humans, monkeys, dogs, rabbits, guinea pigs and rodents (Bockman and Cooper, 1973; Owen and Jones, 1974; Owen and Nemanic, 1978; Trier, 1991). They have a unique structural morphology. The apical surface is covered with a variable number of irregular microvilli which are shorter, wider and fewer in number than exhibited by the neighbouring enterocytes (Owen and Jones, 1974; Owen and Nemanic, 1978; Smith and Peacock, 1980). The presence of numerous endocytotic vesicles, capable of transporting macromolecular tracers such as horseradish peroxidase (Owen, 1977) and ferritin (Bockman and Cooper, 1973; Bye, Allan and Trier, 1984) is evident. The transport of macromolecular substances, such as cationised ferritin, certain lectins and the B subunit of cholera toxin, from the intestinal lumen to the basolateral

membrane of the M cell or the subepithelial intercellular space has been reported to occur within minutes by adsorptive endocytosis (Owen, 1977; Bye, Allan and Trier, 1984; Neutra, Phillips, Mayer and Fishkind, 1987). Macromolecules, such as albumin, that do not adhere to the apical surface of M cells may also cross the epithelial barrier via the M cell by fluid-phase endocytosis, although this process has a markedly reduced efficiency compared with adsorptive endocytosis (Neutra *et al*, 1987). In a review of the current literature, Trier (1991) concluded that virtually any component of the intestinal luminal contents may be transported across the mucosa by this means, if that component is resistant to enzymatic hydrolysis.

With this in mind, it is perhaps not inconceivable to suggest that the differences in the permeation of G-CA, demonstrated in the ileum and jejunum, are attributable to the differences in the morphology of the small intestine, and more specifically to the M cells of the ileum. If the coupling of tetragastrin with cholic acid in some way results in an increase in the stability of tetragastrin compared with the native tetragastrin (which is likely to undergo considerable proteolysis), this may be a feasible proposal. Nevertheless, the possibility remains to be fully resolved.

4.4.3 Hormonal release

The likelihood that ileal instillation of G-CA evokes an increase in gastric acid secretion through the release of a hormone(s) which ultimately results in the stimulation of the parietal cells must be considered. We are able to rule out mediation through a vago-vagal reflex since ligation of the oesophagus at its junction with the stomach (see Methods), includes the anterior and posterior branches of the vagus nerves which run over the surface of the oesophagus, with the result that these nerves are blocked. This has been confirmed experimentally (Morrison, personal communication). An intrinsic reflex mediated through the gastrointestinal tract may also be excluded. Apart from the very considerable distance from the ileum to the stomach, and the presence of the hyponeural segment between the duodenum and the antrum, ligation of the stomach tube at the gastroduodenal junction would further rule out this possibility.

A review of the literature has not revealed any evidence that ileal instillation of foodstuffs leads to stimulation of gastric acid secretion. Indeed, the converse seems to be true. In human volunteers, ileal perfusion of carbohydrates and lipids resulted in

decreased gastric acid output, whereas ileal perfusion of peptones had very little effect (Layer, Holst, Grandt and Goebell, 1995). The reduced gastric acid secretion was closely correlated with an increase in the levels of glucagon-like peptide-1 (GLP-1). Similarly, in the anaesthetised dog, ileal infusion of bile induced a significant decrease in gastric acid secretion, which was accompanied by elevated plasma concentrations of gut glucagon-like immunoreactivity (gut GLI) (Namba, Matsuyama, Itoh, Imai, Horie and Tarui, 1986). This response bears a remarkable parallelism with the "ileal brake" mechanism, in which ileal infusion of fatty acids, triglycerides and complex carbohydrates delays gastric emptying and intestinal transit time (Holgate and Read, 1985; Fone, Horowitz, Read, Dent and Maddox, 1990; Lin, Doty, Reedy and Meyer, 1990; Pironi, Stanghellini, Miglioli *et al*, 1993), thereby constituting a negative feedback mechanism. In humans and dogs, instillation of mixed nutrients into the ileum resulted in the reduction of gastric emptying, which was closely correlated with plasma levels of Peptide YY (Pironi *et al*, 1993; Wen, Phillips, Sarr, Kost and Holst, 1995). In addition, GLP-1 may play a small role in this reflex, in the dog at least (Wen *et al*, 1995).

The outcome of such studies would, thus, appear to indicate that the response to ileally-administered G-CA is unlikely to occur as a result of a known hormonal mechanism. Otherwise, the only other possibility is that a hitherto unreported hormonal action underlies the responses caused by G-CA.

4.5 Consideration of the mechanisms involved in ileal tetragastrin-induced gastric acid secretion

A surprising outcome of this study was that, after ileal administration of G-CA (600μg kg^{-1}), there did arise a measurable increase in gastric acid secretion in response to instillation of tetragastrin (3000μg kg^{-1}) into the ileum. This was a striking effect, recorded in seven rats, which resulted in a mean increase in gastric acid secretion above baseline of 3.05 ± 1.89μmol $180min^{-1}$. (The mean increases ± S.D. in gastric acid output, presented in Table 3-7, are reproduced in Table 4-4). In attempting to explain these results, several possibilities must be considered.

Table 4-4: Mean peak and cumulative (cum.) increases (± S.D.) in gastric acid secretion in response to ileally-administered tetragastrin-cholic acid conjugate (G-CA) (600μg kg^{-1} in 1.0ml saline) over 180 minute period, subsequent ileally-administered tetragastrin (G4) over 180 minute period, and intravenous G-CA (15μg kg^{-1}) as a first and final procedure (restatement of Table 3-7).

	Initial I.V. G-CA		Ileal G-CA	Ileal G4	Second I.V. G-CA	
	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})	Cum. increase (μmol $180min^{-1}$)	Cum. increase (μmol $180min^{-1}$)	Peak increase (μmol $15min^{-1}$)	Cum. increase (μmol hr^{-1})
Mean ± S.D.	0.57±0.24 (P=0.001)	1.09±0.52 (P=0.004)	1.31±0.44 (P=0.003)	3.05±1.89 (P=0.006)	0.70±0.42 (P=0.005)	1.15±0.59 (P=0.003)

P-values denote statistical significance for comparison with zero baseline.

4.5.1 Permeability changes caused by G-CA

The possibility that G-CA caused a lasting permeability change in the ileal mucosa which, thus, allowed permeation by tetragastrin must be considered. The increase in gastric acid secretion evoked by intra-ileal tetragastrin above baseline levels was generally prolonged over a period of between 90 and 105 minutes, although in one rat, gastric acid levels remained elevated above baseline for considerably longer (225 minutes). The corollary of these experiments is that the absence of a response to ileally-infused tetragastrin, without the conditioning effect of G-CA, could not be attributed to the proteolytic degradation of tetragastrin within the intestinal lumen since, when injected after intra-ileal G-CA, intra-ileal tetragastrin did cause increased gastric acid secretion. Rather, it suggests that the barrier provided by the intestinal wall contributes more, in the former case, to the absence of a response. Thus, there appears to be a *prima facie* case that G-CA caused a long lasting permeability change which allowed tetragastrin to cross the mucosal barrier.

However, light microscopic (LM) analysis of the G-CA-perfused ileal wall (see Figs. 3-22C and D) revealed little evidence of structural differences, when compared with LM analysis of the control mucosa, excised from a separate animal in which perfusion of the ileum was not carried out (see Figs. 3-22A and B). This suggests that a pathological change of the ileal mucosa is not involved, at least at the light microscopic level.

Additional support for this view comes from the results obtained from our investigation of the response to jejunally-perfused G-CA (600μg kg^{-1}), in which no measurable increase in gastric acid output was recorded. If the effect of G-CA occurs via a permeability increase, for example, at the side of the tight junctions between enterocytes, one might have expected, intuitively, that G-CA would also permeate across the jejunum. Thus, we are confident that, on the basis of the present results, G-CA has had no discernible effect on the permeability of this mucosa.

4.5.2 Potentiation of subthreshold absorption of tetragastrin

The control experiments which reported an absence of any increase in gastric acid secretion when tetragastrin was infused intra-ileally (Section 3.1.1) could not exclude the possibility that tetragastrin had, in fact, been absorbed but in amounts which were insufficient to stimulate gastric acid secretion.

The mechanism by which ileal tetragastrin, subsequent to administration of ileal G-CA, elicited an increase in gastric output may thus have arisen as a consequence of potentiation of this low level of intestinal absorption of tetragastrin by the continued presence of subthreshold doses of ileal G-CA in the circulation. However, this is unlikely since the increase in gastric acid levels evoked by the second intravenous injection of G-CA, administered subsequent to ileal G-CA, demonstrated no significant potentiation of either the peak or the cumulative response to the first I.V. injection of G-CA, as a first procedure (P=0.63 and P=0.51, respectively) (see Section 3.1.3(ii)).

4.5.3 Release of G-CA adsorbed by the ileal mucosa

The adsorption of drugs by the intestinal mucosa has been well documented (Barry and Braybrooks, 1975; Nimmerfall and Rosenthaler, 1980; Shurgers, De Blacy and Crommelin, 1985; Niibuchi, Aramaki and Tsuchiya, 1986). Accordingly, it is conceivable that, in the present study, the ileal injection of tetragastrin acted to release any mucosal G-CA, injected previously, which had been adsorbed by the mucosa of the ileum. This implies that it was the administration of a second infusion into the ileum, rather than what it contained, that led to the elevated gastric acid secretion. The increase in gastric acid levels above baseline would thus be explained if

subsequent transportation of the residual G-CA across the ileal mucosa, via the bile salt active carrier system, into the circulation had occurred.

4.5.4 Hepatic recirculation of G-CA

It is also possible that the elevation of gastric acid levels which were recorded in response to ileal tetragastrin, administered subsequent to ileal G-CA, is instead associated with the recycling of ileal G-CA via the enterohepatic circulation. In other words, the infusion of fluid into the ileum facilitated the rate at which G-CA, excreted into the duodenum, arrived at the ileum. The magnitude of such a process would, however, depend on the rate of excretion of hepatic G-CA which, in turn, would be dependent on the rate of uptake of G-CA by the hepatocytes. In addition, the process would depend on the rate of inactivation of G-CA within the hepatocytes. In the present study, the increase in gastric acid levels evoked by a second intravenous injection of G-CA, administered subsequent to ileal G-CA, demonstrated no significant potentiation of the peak or cumulative response to the first intravenous injection of G-CA, as a first procedure ($P=0.63$ and $P=0.51$, respectively) (see Section 3.1.3(ii)), which makes it unlikely that re-circulated G-CA played a role in this series of experiments.

Even if recycled G-CA, *per se*, were insufficient to account for the elevated gastric acid secretion arising when intra-ileal tetragastrin was infused, there may be another consequence, even if this process occurred at a subliminal level. If we surmise that ileally-administered tetragastrin does in fact manage to permeate the intestinal mucosa, but is cleared rapidly from the portal blood by the liver, this would lead to an absence of response, as seen in our control experiments (Section 3.1.1). This being the case, pre-injection of ileal G-CA, which may also be taken up by hepatocytes as well as circulating in the plasma, may result in the saturation of those sinusoidal membrane carrier proteins of the hepatocytes involved in hormonal clearance from the portal circulation. As a result, subsequently administered ileal tetragastrin may now be subjected to the diminished effects of hepatic first-pass metabolism, thereby enabling the entry of tetragastrin into the systemic circulation to produce elevation of gastric acid secretion above basal levels.

4.5.5 Sparing of G-CA from proteolytic degradation by tetragastrin

An alternative explanation for the observed increase in gastric acid levels following the injection of tetragastrin into the ileal lumen subsequent to the ileal administration of G-CA, is that the recirculation of G-CA through the liver and bile duct does occur, and that tetragastrin is unable to permeate the ileal mucosa. However, proteolytic degradation of this luminal tetragastrin may, thus, spare the proteolytic degradation of recirculated G-CA or subliminal residual amounts of G-CA. However, it seems unlikely that this mechanism alone could account for the sizeable increase in gastric acid secretion which was measured following ileal administration of tetragastrin, compared with the smaller increase observed in response to ileal G-CA (Table 4-4). Nevertheless, there is the possibility that summation of this action with the flushing of adsorbed G-CA by injection of tetragastrin, may account for the increase in gastric acid secretion observed in this study.

4.6 Time course of responses to ileal tetragastrin and G-CA

The time courses of the responses to ileal G-CA and ileal tetragastrin (when preceded by ileal G-CA) were very similar in that gastric acid levels remained elevated above baseline for an extended duration of between 90 and 130 mins. Slight variations were observed in the occurrence of the peak increase in gastric acid levels. In the case of ileal G-CA, the peak level arose between 60 and 105 mins after administration, whereas, with ileal tetragastrin (when preceded by ileal G-CA), a peak increase was recorded earlier at between 15 and 60 minutes following instillation.

The time course of the increase in gastric acid secretion (e.g. Figs. 3-8, 3-11 and 3-16) imparts rather little information regarding the mechanism behind the absorption of ileal G-CA. The absence of intestinal carriers specific to the transport of tetrapeptides (as reviewed by Ganapathy *et al*, 1994) indicates that the movement of tetragastrin across the ileal mucosa could occur only by diffusion which would be driven by a concentration gradient. As shown by the absence of a response to intra-jejunal infusion of tetragastrin (and of G-CA), this route would seem to be of minimal importance. The transport of G-CA would thus seem to depend upon the operation of a facilitated transport mechanism, namely the bile salt transport protein. In this case, one perspective may be that operation of a facilitated transport system would be expected to remove G-CA from the ileum at a relatively fast rate, which would be at

variance with the observed time course of the response. On the other hand, the ileal bile salt transport system is considered to operate maximally at a low concentration (M^{c}Clintock and Shiau, 1983). Since we instilled a relatively large amount of G-CA into the ileum, it is conceivable that the transport of bile salts might have occurred at a low rate over an extended time period. Thus, no unequivocal inferences may be drawn from the time courses of the responses to intra-ileal infusions.

4.7 Further studies

In order to seek answers to the unresolved issues raised by this study, several further lines of investigation may be undertaken.

4.7.1 Bile acid transport inhibitors

Bile acid reabsorption inhibitors (BARI) have been reported to inhibit specifically, and in a concentration dependent manner, the uptake of [^{3}H]-taurocholate into rabbit ileal brush border membrane vesicles and in a rat ileum perfusion preparation, *in situ* (Wess, Kramer, Enhsen *et al*, 1994; Kramer, Wess, Baringhaus *et al*, 1995). Measurement of the gastric acid output in the presence of such inhibitors would allow us to state rather more conclusively whether the mechanism underlying ileal transmucosal transport of G-CA involves the bile salt transporters. For completion, the study would need to demonstrate a reversibility of any inhibitory effect, that is, after washing out the BARI, we would need to be able to show that absorption of ileally-infused G-CA was reinstated.

4.7.2 Increasing the length of the conjugated peptide

Reports have indicated that increasing the chain length of the peptide attached to the C-3 position of the steroid nucleus of cholic acid results in a diminished affinity for the ileal bile acid transporter (Kramer *et al*, 1994). Peptide-cholic acid conjugates with up to four amino acid residues in the peptide side chain were able to competitively inhibit the uptake of [^{3}H]-taurocholate in a dose-dependent manner. However, coupling of eight amino acid residues with cholic acid resulted in a significantly reduced affinity for the ileal bile salt carrier, thus allowing greater absorption of [^{3}H]-taurocholate. Moreover, although the conjugation of a heptapeptide renin inhibitor to cholic and taurocholic acid at the C-3 position

demonstrated high levels of affinity for the bile salt transporter in Caco-2 cells, the conjugate was not transported by the ileal bile salt transporter *in vivo* (Kim *et al*, 1993). This is of considerable importance, since peptides employed as therapeutic agents are often in excess of seven or eight amino acids; insulin, for example, is composed of 51 amino acids.

By conjugating cholic acid with a peptide chain that is comprised of an increased number of amino acid residues making up the carboxy terminal end of gastrin, for example, pentagastrin (G5) or heptadecapeptide gastrin (G17), we could repeat the experimental protocols carried out in the present study. This would allow us to determine the effect that peptide length has on the transport of peptide-cholic acid conjugates in the ileum.

4.7.3 Determination of the mechanism(s) involved in ileal tetragastrin-induced gastric acid secretion

4.7.3(i) *Permeability changes caused by G-CA*

The pre-conditioning of the ileum by G-CA to allow the possible ileal permeation of subsequently infused tetragastrin requires further elucidation. It would thus be of interest to determine the degree to which permeation of other hormones are enhanced by the employment of a different *in vivo* bioassay. For example, secretin (I.V.) has been shown to be very potent in evoking a marked increase in the secretion of HCO_3^- and protein in pancreatic juice of rats (Wheeler, Eardley, M°Nulty, Sutcliffe and Morrison, 1997). The ileal instillation of secretin, subsequent to ileal administration of G-CA, and the measurement of the resultant HCO_3^- and protein in pancreatic secretions, relative to control levels, would provide an indication of the degree to which permeability of the ileum to macromolecules is altered.

Additionally, histological analysis of the perfused ileum, comparative to the unperfused ileum, may be further extended by the use of transmission electron microscopy to examine the integrity of the epithelium with specific attention paid to the integrity of the tight junctions between cells.

4.7.3(ii) *Displacement of adsorbed G-CA from ileal mucous layer*

The possible involvement of adsorbed residual G-CA, displaced from the mucous layer of the ileum by injection of 1.0ml tetragastrin solution into the ileal lumen, in the

induction of increased levels of gastric acid secretion, may be resolved by replacing the ileal tetragastrin injection with ileal injection of the same volume of isotonic saline; a resultant increase in gastric acid levels would provide confirmation of this flushing effect.

Alternatively, determination of the presence of residual G-CA in the ileum may be achieved by assay of the luminal contents using a photometric method, specific for conjugated and unconjugated cholates, as described by Irvin, Johnston and Kopala (1944).

4.7.3(iii) *Hepatic recycling of G-CA*

It would be of interest to ascertain whether the increase in gastric acid secretion presented over the 180 minutes following ileal injection of tetragastrin contains a component due to recycling of G-CA via the enterohepatic circulation. This may be determined by repetition of the experiments in rats in which the bile duct has been ligated or the flow of bile diverted to the exterior by a cannula, thereby excluding the possible re-entry of G-CA into the lumen of the ileum.

4.8 Conclusions

In the present study, we have demonstrated the facilitation of ileal absorption of tetragastrin through the coupling of tetragastrin with cholic acid at the C-24 position. The absorption of ileal G-CA is unlikely to be attributable to passive diffusion of the non-ionised, lipophilic form of molecules since the compound was estimated to be in a predominantly ionised form. For transport to occur at a substantial rate, this would imply that a facilitated transport mechanism was involved. Moreover, this was a response specific to the ileum, since no apparent intestinal transmucosal movement of G-CA was observed after instillation of G-CA into the jejunum. Although we must consider, as possible mechanisms, the role played by the M cells of the ileum, as well as initiation of a hormonal release mechanism, the most likely explanation for the permeation of the ileal wall is transport of the conjugate by the bile acid carrier system, which is present only in the ileum. If this is the case, the results of this study are very encouraging in terms of a successful approach to address the improvement of oral absorption of otherwise poorly absorbed macro-molecules.

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APPENDIX

6.1 Ionisation states of intestinally-administered agents

In general, the unionised form of a weak acid or base will penetrate the intestinal wall at a faster rate than the corresponding ionised acid or base. The ratio of ionised and unionised form of a weak acid or weak base in a solution may be calculated using equation 6-1, if we know both the pH and the pKa values. When pH equals pKa, the compound is 50% ionised.

$$pH = pKa + \log \frac{[A^-]}{[HA]} \qquad (HA \rightleftharpoons H^+ + A^-)$$

equation 6-1

Determination of the state of ionisation of complex molecules, such as tetragastrin and the tetragastrin-cholic acid conjugate (in which the pKa values relating to the entire molecule are unknown) was made from a consideration of the ionisation of the carboxyl group (COOH, part of aspartate in both compounds) and the primary and secondary amine groups (NH_2 and NH, respectively). The pKa values for these (taken from Mahler and Cordes, 1969b) are listed in Table 6-1. The pH of the tetragastrin and tetragastrin-cholic acid conjugate solutions, as used in the experimental procedures, were measured prior to intestinal infusion, and are also presented in Table 6-1.

Table 6-1: pH values of intestinally administered solutions (in 1.0ml isotonic saline), and the pKa values of the carboxyl and primary and secondary amine side groups of these compounds.

Compound	Side groups	pKa	pH of solution
Tetragastrin	COOH	4.6	3.8
	NH_2	7.9	
	NH	7.0	
Tetragastrin-cholic acid conjugate	COOH	4.6	4.7
	NH_2	7.9	
	NH	7.0	

6.1.1 Tetragastrin

6.1.1(i) *Carboxyl group*

$$COOH \rightleftharpoons COO^- + H^+$$

$$3.8 = 4.6 + \log \frac{[COO^-]}{[COOH]}$$

$$0.8 = \log \frac{[COOH]}{[COO^-]}$$

$$6.3\,[COO^-] = [COOH]$$

The carboxyl group of tetragastrin is approximately 14% ionised.

6.1.1(ii) *Primary amine group*

$$NH_3^+ \rightleftharpoons NH_2 + H^+$$

$$3.8 = 7.9 + \log \frac{[NH_2]}{[NH_3^+]}$$

$$4.1 = \log \frac{[NH_3^+]}{[NH_2]}$$

$$12589\,[NH_2] = [NH_3^+]$$

The primary amine group of tetragastrin is approximately 100% ionised.

6.1.1(iii) *Secondary amine group*

$$NH_2^+ \rightleftharpoons NH + H^+$$

$$3.8 = 7.0 + \log \frac{[NH]}{[NH_2^+]}$$

$$3.2 = \log \frac{[NH_2^+]}{[NH]}$$

$$1584\,[NH] = [NH_2^+]$$

The secondary amine group of tetragastrin is approximately 100% ionised.

6.1.2. Tetragastrin-cholic acid conjugate

6.1.2(i) *Carboxyl group*

$$COOH \rightleftharpoons COO^- + H^+$$

$$4.7 = 4.6 + \log \frac{[COO^-]}{[COOH]}$$

$$0.1 = \log \frac{[COO^-]}{[COOH]}$$

$$1.3\,[COOH] = [COO^-]$$

The carboxyl group of tetragastrin-cholic acid conjugate is approximately 57% ionised.

6.1.2(ii) *Primary amine group*

$$NH_3^+ \rightleftharpoons NH_2 + H^+$$

$$4.7 = 7.9 + \log \frac{[NH_2]}{[NH_3^+]}$$

$$3.2 = \log \frac{[NH_3^+]}{[NH_2]}$$

$$1585\,[NH_2] = [NH_3^+]$$

The primary amine group of tetragastrin-cholic acid conjugate is approximately 100% ionised.

6.1.2(iii) *Secondary amine group*

$$NH_2^+ \rightleftharpoons NH + H^+$$

$$4.7 = 7.0 + \log \frac{[NH]}{[NH_2^+]}$$

$$2.3 = \log \frac{[NH_2^+]}{[NH]}$$

$$199\,[NH] = [NH_2^+]$$

The secondary amine group of tetragastrin-cholic acid conjugate is approximately 100% ionised.

You know, Tolstoy, like myself, wasn't taken in by superstitions like science and medicine.

George Bernard Shaw

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