THE MECHANISM OF A DRUG-INDUCED GASTROINTESTINAL TOXICITY

BY

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Summary

The orally-active, anti-allergic, chromone drug, proxicromil, (6,7,8,9-tetrahydro-5-hydroxy-4-oxo-10-propyl-4H-1-naphtho[2,3-b] pyran-2-carboxylate), produced an unexpectedly high incidence and severity of gastro-intestinal disorders in humans. These side-effects had not been predicted following a complete safety evaluation programme in animals. The purpose of the work described in the following chapters was to elucidate the mechanism by which proxicromil caused gastro-intestinal disorders in man, to determine whether the effect in humans could have been predicted from animal studies and, if so, to develop a simple but effective toxicity study that could be used with future drugs to predict their potential to induce this type of side-effect in humans.

The gastrointestinal effects of proxicromil were found to be associated with delayed gastric emptying, and a method was developed in rats for demonstrating the potential of a candidate compound to cause an inhibition of gastric emptying. The method was shown to be reproducible and to detect the effect of 'standard' drugs such as codeine phosphate (decreases in gastric emptying) and metoclopramide (increases). A secondary screen used to evaluate drugs shown to be active in the primary test and to screen out false-positives was developed in marmosets. X-ray photography after a barium meal also demonstrated inhibition of gastric emptying. Proxicromil was shown to be a potent inhibitor of gastric emptying in both the rat primary screen and the marmoset secondary screen.

Inhibition of gastric emptying was associated with a much lower and flatter plasma drug concentration v time course than was the case
where inhibition did not occur. This effect has consequences on the relevance of toxicity studies in different species.

From measurements of stomach pressure changes in spontaneously contracting rat stomachs and spontaneous contractions of rat fundic strip preparations, it was concluded that proxicromil had no direct effect on the musculature of the stomach. The drug was also shown to have no direct activity on the pyloric sphincter.

An in situ preparation, developed to enable recordings of spontaneous contractions of the stomach to be made whilst retaining both nervous and blood supplies, showed that proxicromil had no effect when administered directly into the stomach. However when administered to the duodenum the drug induced a dramatic and immediate cessation of stomach contractions. It was demonstrated that the drug exerted its inhibitory activity on gastric emptying by a reflex mechanism initiated in the first 20 to 30 cm of the rat duodenum, that the effect occurred immediately when the compound reached the duodenum and that the duration of activity was related to the concentration of drug in the duodenum.

The relative inhibitory activities of proxicromil on gastric emptying after oral and intravenous administration indicated that absorption was not necessary for activity. The time of onset of the activity, the lack of activity in rats with a common blood circulation to proxicromil treated animals, and the inhibitory effect of a local anaesthetic in the duodenum, all demonstrated that the drug exerted its inhibitory activity via a nervous rather than a hormonal pathway. It was demonstrated that the reflex was probably centrally-mediated via a non-cholinergic component of the vagus nerve.
Structure-activity studies with a range of chromones and other drugs, from the completely inactive sodium cromoglycate to the very potent proxicromil, indicated that certain groupings on the chromone nucleus could be expected to increase or decrease the potential of a new chemical entity to induce similar gastro-intestinal disorders in humans. A positive correlation between inhibition of gastric emptying and sensory irritancy in the lung was demonstrated, indicating that the activity of proxicromil in the duodenum may be associated with its irritant properties. Thus the drug may initiate a reflex in the duodenum that has a physiological role in protecting the intestine from irritant or otherwise noxious substances. An alternative explanation to this arose as a result of the finding that bile salts were potent inhibitors of gastric emptying and that many of the active chromones, particularly proxicromil, have high surface tension properties. These results lead to the suggestion that proxicromil may exert its activity by stimulating a reflex that has a physiological role in responding to bile salts to regulate the rate at which fats enter the duodenum.

In conclusion, this research has shown that the gastro-intestinal side-effects that occurred in humans with this drug could have been predicted from special animal studies. Animal toxicity tests were developed that could potentially predict the effect in man of future drugs of this class. Structure-activity studies indicated certain groupings on the chromone nucleus that are likely to increase the potential to produce gastro-intestinal side-effects in man. A combination of the knowledge gained from the structure-activity studies and the use of the animal tests developed as screens for this type of activity, should prevent drugs with this type of side-effect from being developed to an advanced stage.
CHAPTER 1

INTRODUCTION
Occasionally, in clinical practice, marketed drugs induce toxicity that had not been predicted from animal studies. Unpredicted side-effects can vary from minor problems that are acceptable within a risk/benefit context, such as the diarrhoea or constipation following chlorpromazine treatment, to the unacceptable effects of thalidomide.

Historically, the science of toxicology has always lagged behind the development of efficacious drugs. In 1949 for example, chloramphenicol was introduced as an anti-microbial substance produced initially from the growth of Streptomyces venezuelae. As well as inducing some minor and relatively infrequent side-effects, (although blindness due to damage of the optic nerve has also been attributed to the drug), (Meyler, 1966; Meyler & Herxheimer, 1968), chloramphenicol induced serious blood dyscrasias in several hundreds of patients, particularly children, of which the majority of cases were fatal (Dillon et al., 1964; Brit. Med. J., 1967; Garrod, 1964). Toxicity studies in animals in 1949 were rare, the emphasis being on efficacy.

Chloramphenicol was probably the first major medical disaster in modern medicine. The occurrence was excused on the grounds that no-one expected such an effect and that it could therefore not have been foreseen. The medical profession however was awakened to the possibility of serious adverse effects induced by otherwise useful and efficacious drugs.
The second major disaster however came with as much surprise as had the first. Thalidomide, introduced in 1955 as an extremely safe sedative was withdrawn from marketing in 1961 after its now well-known teratogenic effects had become apparent. Teratogenicity, or even placental transfer, of drugs were concepts that had not at that time been considered to have relevance to apparently safe drugs. That neither the total dose nor the duration of administration were of much influence on the incidence of congenital malformations were also novel concepts; the critical factor being that the drug, even possibly a single tablet, had been taken during the first trimester, particularly between the 27th and 40th days of pregnancy (Mellin & Katzenstein, 1962). Chloramphenicol may have stirred the surface of toxicology; thalidomide resulted in action.

There have been few major disasters to compare with thalidomide or chloramphenicol since the 1960s (practolol perhaps being the most notorious exception), due to both the much more vigorous testing by the pharmaceutical companies and the close scrutiny and approval required by Regulatory Authorities commencing in the UK with the formation of the Dunlop Committee. It is unlikely that another potent teratogen or a potent bone-marrow depressant will ever be marketed. However, it is unacceptable to be reasonably confident of this for each physiological function, only after a serious medical crisis has occurred. Toxicologists are occasionally reminded of the possibility of serious problems in man; the most recent being the withdrawal in 1983 of benoxaprofen (Opren), the pharmacokinetics of which had not been adequately studied in ageing animals. The toxicity of this drug in old patients verged on being a major disaster, and was saved from being so only by post-marketing observations and surveillance, not
by toxicity studies in animals as should have been the case. Once again this particular door should now be closed.

The lessons are therefore there to be learned, not only that major disasters may occur but also that less dramatic side-effects are not infrequent. The occurrence of minor side-effects necessitates taking into account the risk/benefit ratio to meet an acceptable risk to the patient for the disease or condition being treated (Pochin, 1981). A number of side-effects however have led directly to the use of drugs for conditions other than those for which they were originally introduced (Breckenridge, 1981). (See Table 1.1).

Table 1.1 Major disease entities treated by drugs not introduced for that purpose

<table>
<thead>
<tr>
<th>Drug</th>
<th>Condition treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol</td>
<td>Hypertension</td>
</tr>
<tr>
<td>Sulphinpyrazone</td>
<td>Ischaemic heart disease</td>
</tr>
<tr>
<td>Diazepam</td>
<td>Status epilepticus</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>Epilepsy</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>Schizophrenia</td>
</tr>
<tr>
<td>Oestrogens/Progestogens</td>
<td>Contraception</td>
</tr>
<tr>
<td>Imipramine</td>
<td>Depression</td>
</tr>
<tr>
<td>Probenecid</td>
<td>Gout</td>
</tr>
<tr>
<td>Lignocaine</td>
<td>Arrhythmias</td>
</tr>
</tbody>
</table>

(From Breckenridge, 1981)

However, although side-effects per se may not necessarily be detrimental, it should be the aim of the toxicologist at least to predict them.
Although the introduction of regulatory requirements has undoubtedly been largely responsible for preventing many unsafe medicines from being sold (Grahame-Smith, 1981), regulations have not been without some detrimental effects. As a result, primarily of the thalidomide disaster, the number of toxicity studies, the duration of those studies, and their cost, have risen substantially. The design of some of these studies were, in retrospect, ill-conceived (see for example Zbinden, 1982; Tattersall, 1982, 1983) but have nevertheless become established as normal requirements.

Although virtually all regulatory statements are claimed to be only guidelines, the pharmaceutical companies are faced with the dilemma of risking a delay of perhaps 12 months or more in marketing a new drug. This can occur if the product license application is rejected because the company has not carried out a study which they believed, rightly or wrongly, was scientifically unjustified. A twelve month delay can result in a financial loss of four to six million pounds (Christensen, 1981). The tendency therefore is to do all recommended studies. This is expensive and time-consuming and reduces the opportunity to explore new, perhaps more relevant studies. A solution to these contradictory aims ought gradually to evolve as international regulations become more consistent, a freer communication between the pharmaceutical companies and the authorities develops, and toxicology itself concentrates more on individual organ functions in more predictive studies.

Furthermore, it is difficult to predict that a drug will be free from side-effects just because an analogue is known to be free from toxicity. Intal (sodium cromoglycate) despite 15 years of extensive clinical use world-wide, has not been reported to induce any
consistent side-effects, but it would be unjustified to assume that because of this, all chromone compounds can be considered to be safe. As a class of chemicals they certainly appear to induce less toxic reactions in animals than many others, but each individual chromone has the potential to induce a toxic response. Proxicromil is one such compound.

Proxicromil, (6,7,8,9-tetrahydro-5-hydroxy-4-oxo-10-propyl-4H-1-naphtho[2,3-b]pyran-2-carboxylate) a chromone carboxylic acid derivative that has anti-allergic properties, was found to induce such a high incidence of gastro-intestinal side-effects in humans that withdrawal of the drug was considered. These side-effects had not been predicted from animal studies.

The purpose of the work described in the following chapters was to elucidate the mechanism by which proxicromil caused gastro-intestinal disorders, to determine whether the effect in humans could have been predicted from animal studies and, if so, to develop a simple but effective toxicity study that could be used with future drugs to predict their potential to induce this type of side-effect in humans. The initial hypothesis on which this work was based was that the gastro-intestinal side-effects which occurred in humans were due to an inhibition of gastric emptying. This hypothesis arose from a consideration of the symptoms, particularly nausea, (see page 50), and their time course after administration of the drug and their similarity to symptoms which occur shortly before and during the nausea and vomiting accompanying gastric infection. In such circumstances vomitus invariably contains food consumed many hours previously, indicating a marked inhibition of gastric emptying that must have occurred prior to the nausea, and possibly initiated it.
Physiology of the stomach - secretory function

The principal functions of the gastro-intestinal tract are secretory, absorptive and propulsive. The gastro-intestinal tract is also an organ of excretion. No other organ, or group of organs, comes into contact with the large variety of externally applied insults as does the gastro-intestinal tract. Whatever the content of the food or liquid consumed, however concentrated or noxious it may be, the gastro-intestinal tract is structured so that by the time the ingested material has proceeded only a short distance along the duodenum it has been altered so that its pH, fat content, protein content, energy content, noxious or irritant content etc. have all been adjusted to within quite narrow, acceptable ranges. The stomach, although generally considered as a receiving and storage organ, is much more important as a major homeostatic organ. The duodenum provides information concerning its food content and the stomach responds by retaining or allowing food through to the intestine.

The alimentary tract produces both exocrine and endocrine secretions. Exocrine secretions begin with the saliva. The salivary glands appear to be unique within the gastro-intestinal tract by being under the exclusive control of nervous mechanisms, with parasympathetic stimulation causing both secretion and vasodilation. Sympathetic neurones releasing noradrenaline also innervate the salivary glands causing secretion of a more viscous saliva and vasoconstriction although Emmelin (1968) contested such a conclusion.

In the stomach, the digestive enzyme pepsin, is secreted along with the gastric juice which is produced from the mucus, oxyntic and Chief cells. As secretion increases, the sodium ion concentration falls and
the hydrogen ion concentration rises. The surface of the epithelium is lined by mucus glands which also line the rugae and gastric pits, See figure 1.1, and produce mucus that contains a variety of mucopolysaccharides and mucoproteins and the intrinsic factor necessary for the absorption of vitamin B12.

![Diagram of the gastric wall](image)

**Figure 1.1** Schematic representation of the gastric wall showing the muscle layers and nerve complexes (From Hally & Lloyd, 1968).

The oxyntic, or parietal, cells secrete hydrochloric acid and the Chief, or zymogen, cells secrete pepsinogen. Both these cell types occur in the fundic region of the stomach (Oi et al., 1959). The production of hydrochloric acid by the parietal cells works against a concentration gradient of up to $10^6$, the energy being derived from adenosine triphosphate. One theory for the generation of hydrochloric acid involves the formation of carbonic acid by the hydration of carbon dioxide through the action of carbonic anhydrase (see figure 1.2). The bicarbonate ion so formed is removed in the venous blood. The carbonic acid acts as a hydrogen donor, neutralising the hydroxyl
ion that results from the hydrolysis of water. The remaining hydrogen ion from the water is secreted in the gastric juice coupled with chloride transported by an active process. The ambient $\text{HCO}_3^-$ plays an important role in the protective mechanisms of gastric mucosa against autodigestion (Cummins et al., 1948; Kivilaakso, 1981), by secretion into the gastric mucus barrier, and by acting as an intracellular buffer (Kivilaakso, 1981, 1983).

Figure 1.2 Production of hydrogen ions in parietal cells from carbon dioxide and water.

When food is eaten, gastrin production is initiated from enterochromaffin type of cells (G cells) located in the pyloric antrum (McGuigan, 1968), by a number of mechanisms, See Figure 1.3. These are principally, centrally mediated vagal stimulation, gastric
distension and chemical stimulation by amino acids, peptides etc. in the food.

Mechanical stimuli (eg. distension - food) 
Vagal stimulation
Chemical stimuli (eg. amino acids)

+ Acid
- Atropine

Gastrin releasing cells

+ - Ganglion blocking drugs

Figure 1.3 Factors causing stimulation (+) and inhibition (-) of gastrin from G cells (From Elwin & Uvnas, 1966).

Lichtenberger and his colleagues (1982) have shown that the ability of amino acids to release gastrin is correlated with their lipid solubility in vitro and that amino acid decarboxylation may be a necessary step in gastrin release. Gastrin release induced by vagal stimulation and gastric distension are enhanced by atropine (Feldman et al., 1979; Dockray & Tracy, 1980), indicating that a non-cholinergic vagal stimulation is involved and also that a cholinergic vagal stimulation inhibits the release of gastrin. Bombesin may be this non-cholinergic transmitter. In contrast to this, gastrin stimulation by amino acids is inhibited by atropine.

Gastrin is also released when food enters the intestine, even when the stomach is denervated. Cholecystokinin is a hormone released from the intestine that may be responsible for this subsequent release of gastrin. Although atropine and related anti-cholinergics have been used for many years to reduce gastric secretions, Ito (1981) has shown that there is little evidence that post-ganglionic,
parasympathetic cholinergic fibres actually innervate parietal cells directly. A simplified diagram of the nerve supply to the stomach wall is shown in figure 1.4.

![Intrinsic Reflex Arcs](image)

**Figure 1.4** Simplified diagram showing the major nerve supplies to the stomach wall (From Hally & Lloyd, 1968)

Pirenzipine, a muscarinic receptor antagonist, also decreases gastric secretions and accelerates the healing of peptic ulcers (Jaup et al., 1980; Londong, 1982). Pirenzipine however has selectivity in its action; showing inhibition in the stomach but not, for example, the heart, bladder or eye (Jaup et al., 1980). Hammer & colleagues have suggested that this selectivity is due to two types of muscarinic receptor, designated M1 and M2 (Hammer & Giachetti, 1982). Those in the stomach are of the M1 type.
The rise in gastrin secretion that occurs after vagotomy (Alumets et al., 1980) due to the lack of acid feedback inhibition of gastrin release, demonstrates the effect of one of the many feedback mechanisms under which the gastro-intestinal tract functions.

Gastrin itself does not appear to be the actual stimulator of the oxyntic cells. This final stimulation appears to be due to histamine - synthesised in the gastric mucosa (Soll et al., 1979) - acting on $H_2$ receptor sites (Black et al., 1972), particularly following vagal stimulation (MacIntosh, 1938).

When the stomach contents enter the duodenum, reflexes initiated in the latter organ, not only control the rate of gastric emptying (discussed later) but also control the rate of acid secretion in the stomach. Thus an acidified duodenum reduces both the rate of gastric emptying and the rate of acid secretion. This effect is generally referred to as the intestinal phase of the control of acid secretion.

The major motor component of the stomach consists of a series of peristaltic waves occurring in rhythmic sequence. In man the rhythm is approximately three per minute (Hightower & Code, 1950); in dogs it is four to five per minute (Bass et al., 1961; Carlson et al., 1966) and in rats it is three to five per minute, (This report, Chapter 2). As the contraction moves into the terminal antrum, the pyloric canal also contracts. This results in most of the contents propelled by the contraction being forced back into the stomach, thus mixing and breaking up the stomach contents. Therefore only a limited quantity of food is expelled into the duodenum following each contraction.
Gastro-intestinal hormones

The word hormone was introduced in 1905 by Starling to describe a chemical system that allowed stimuli received at one part of the body to alter activity at a remote site. Numerous hormones have since been discovered but the first, discovered in 1902 by Bayliss and Starling, was secretin. Despite this early start and the plethora of publications ever since, the activity of gastro-intestinal hormones, including that of secretin, still has not been fully elucidated.

The problems associated with determining precisely the role of each identified hormone are partly explained by factors that most investigators are aware of but do not always fully appreciate. One such factor is species variation, and a second, rarely considered, is the possible presence of vestigial hormones or receptors.

Most authors are aware of species differences but few comment on its implications. When species differences are referred to, it is frequently cursory. For example in a paper devoted to whether or not motilin levels are increased or decreased by alkalinisation of the intestine, Mitznegg and co-workers (1976a) mention that although their results show that in man motilin is decreased under these circumstances, other workers (Dryburgh & Brown, 1975) had shown precisely the opposite in dogs. Such a difference clearly may have been of importance in the interpretation of the results, but this was not discussed. It is important that theories regarding the mechanism of the gut hormones should take such species differences into account.
It would be surprising if, amongst the many dozens of hormones that have been implicated in gastro-intestinal functions, some of them, or the receptors and tissues on which they act, are not vestigial. Because a hormone is present in relatively large concentrations it does not mean that it is either functional or essential. One only has to compare the size of the human appendix with that of the human adrenal or pituitary to appreciate that quantity is not proof of utility. Insulin receptors for example are found on lymphocytes that do not respond to insulin (Gavin, 1974). The reason could be that a vestigial condition is present. Species differences may also, in some cases, be explained by variations in the vestigial nature of the various hormonal components.

Thus when an action of a hormone is determined in an animal and the same hormone is found to be present in man, to conclude, by implication or otherwise, that the hormone has that same action in man may be incorrect. In gastro-intestinal research the frequency of assumptions ignoring the possibility of vestigial components and species variation, is very high. For example the opening sentence of the paper by Debas et al. (1977) concerned with human gastro-intestinal hormones, states: "Motilin is the only peptide extracted from the upper small intestine that stimulates motor activity of the gastric fundus. (Brown et al., 1971)." No mention is made of the fact that Brown only referred to dogs. Similarly the opening sentence to the paper by Christofides et al.,(1979b) states: "Motilin has been shown to stimulate gastro-duodenal motor activity (Jennewein et al., 1975) and to accelerate intestinal transit time (Ruppin et al., 1976) in humans." In fact the paper by Jennewein only refers to dogs. These examples illustrate how essential it is to consider species variation, and within that, to be aware of the high
probability that some of the gastro-intestinal hormones are likely to be vestigial.

Gastrin:

Very few of the actions of the gastro-intestinal hormones (gastrin, secretin, vaso-active polypeptide, cytocystokinin, motilin, enkephalins, Substance P) on gastric motility have been shown conclusively to be physiological. One exception to this is the action of gastrin on the antral or distal stomach to induce contractions. Gastrin is released from 'G' cells located in the pylorus (Solcia et al., 1969). It is released by a number of factors, but primarily after the ingestion of food due to the presence of amino acids or their polypeptides (Davenport, 1977). Meal-stimulated gastrin release is atropine-sensitive (Schiller et al., 1981; Artnak et al., 1981), but so also is vagal stimulation which inhibits gastrin release.

The dominant neuronal pathway resulting in gastrin release appears to be a high threshold, non-cholinergic neurone (Schubert et al., 1981; Track et al., 1981). This hypothesis is supported by the findings of Harty et al., (1981), that although both nor adrenaline and carbachol increase gastrin release, noradrenaline is more potent, inducing a greater maximum release and an earlier peak response. Thus the sympathetic neurones may have a major part to play in gastrin release and do not appear to involve β-receptors (Sank et al., 1981).

Released gastrin enters both the antrum of the stomach and the general circulation but these two types of release are independent of one another, i.e. the release of gastrin into the lumen can be abolished without decreasing the release into the circulation (Palmer et al., 1981), where activity depends on intact vagal innervation (Okike & Kelly, 1977).
Gastrin does not affect the gastric emptying of solids and liquids equally. The hormone causes (i) relaxation of the proximal stomach (Wilbur & Kelly, 1974) thus slowing gastric emptying of liquids, (ii) increases the contractility of the antral or distal stomach (Kelly, 1970; Cooke et al., 1972; Gregory & Tracy, 1964; Sanders & Schimmel, 1970) thus increasing gastric emptying and trituration of solids; and (iii) inhibits constriction of the pylorus (Thomas et al., 1979b) thus enhancing gastric emptying of both liquids and solids.

The reciprocal relationship that exists between gastrin and somastatin (Saffouri et al., 1980), each having opposing actions on acid secretion (Bloom et al., 1974), will also result in indirect opposing actions on motility as a result of the changes in the pH of the gastric contents. The mechanisms controlling the secretions of these two hormones are quite different and separate (Martindale et al., 1982).

**Motilin:**

Motilin is also released, and appears in the circulation after the ingestion of a mixed meal (Christofides et al., 1979a), after fat (Christofides et al., 1979a), or after water (Christofides et al., 1979d). Exogenous motilin increases gastric emptying of solids (Christofides et al., 1979b), and liquids (Christofides et al., 1979c), but at low, physiological concentrations stimulation of gastric emptying of fats does not occur (Christofides et al., 1981), thus supporting the hypothesis that physiological motilin has its major effect on the proximal stomach.

Motilin has been shown to stimulate the canine proximal stomach (Pinnington & Wingate, 1981; Debas et al., 1977) and thus to enhance the gastric emptying of liquids. The canine distal stomach also
contracts to motilin (Green et al., 1976), but this has not been shown to be a physiological response. Vagal tone is essential for optimal activity of motilin (Debas et al., 1977) and both motilin release and motilin activity are atropine sensitive (Lee et al., 1981).

The peculiarity concerning motilin is that despite being excitatory it frequently causes a decreased rate of stomach emptying in humans, (Ruppin et al., 1975). The probable explanation for this is that motilin is also excitatory to the duodenum, much more so in fact than it is to the antrum. Thus an unreceptive duodenum and a weakly contracting antrum may result in delayed gastric emptying because of a disturbance in the co-ordination between antrum, pylorus and duodenum (Green et al., 1976).

The rise in motilin levels that occurs in man on acidification of the small intestine has been proposed as an explanation for the gastric activity that occurs (via the release of secretin and bicarbonate ions) on acidification and for the inhibition of motilin release by the output of bicarbonate ions (Mitznegg et al., 1976a). This would, however, have the effect of inhibiting the release of motilin when it was most needed, i.e. when the output of bicarbonate ions was high because of a low duodenal pH. It is much more likely that the release of motilin is stimulated by a lower pH per se, i.e. until the bicarbonate ions have the pH under control motilin release is high, thus inhibiting gastric emptying and hence decreasing the introduction of more acid into the small intestine.
Secretin:

As its name implies, secretin has a gastric secretory function as its primary action, although this function is inhibitory rather than excitatory. Its action on gastric motility is also fundamentally inhibitory, causing relaxation of the proximal and antral stomachs and constriction of the pylorus in dogs, (Valenzuela, 1976; Kelly et al., 1969; Fisher et al., 1973), thus tending to decrease gastric emptying. Contrary to these reports that secretin constricts the pylorus in man which would tend to decrease bile reflux into the stomach, Ivey et al., (1981) report an increased bile reflux after secretin administration to man; this suggests a relaxation of the pylorus.

Indirectly, the anti-secretory activity of secretin, on gastrin- and food-stimulated acid secretion (Johansson et al., 1972; Tumpson & Johnson, 1969), effects motility as a result of the decreased acidity of the gastric contents.

Vasoactive polypeptide

Vasoactive polypeptide (VIP) appears to be a neurotransmitter rather than a local hormone (Angel et al., 1981). It inhibits proximal contractions (Valenzuela, 1976), and relaxes gastric muscle, (Cooke, 1975), antagonises gastrin-induced contractions of the distal stomach (Morgan et al., 1978a) and relaxes pyloric muscle (Edin, 1980). VIP uncouples electro-mechanical coupling during spontaneous and acetylcholine-induced activity and antagonises pentagastrin-induced increases in activity by a different mechanism. Both the force and amplitude of pentagastrin-induced contractions are decreased whereas only the force of acetylcholine-induced and spontaneous contractions are reduced (Morgan et al., 1978a).
Cytocystokinin:

Although cytocystokinin (CCK) is found in the brain, it does not appear to play a role in appetite control (Hansky et al., 1981). It is released after food in man, the greatest response being after protein, possibly due to duodenal acidification following a protein meal (Bernard et al., 1981). CCK relaxes the proximal stomach (Valenzuela, 1976) hence slowing the rate of gastric emptying of liquids (Yamagishi & Debas, 1978). The inhibition of gastric emptying by CCK is one of the exceptions mentioned earlier that have been shown to have a physiological role, at least in dogs (Debas et al., 1975; Strunz & Grossman, 1978). Under the influence of CCK antral peristalsis is enhanced by an increase in the frequency of the pacesetter potential and the number of action potentials in the distal stomach (Morgan et al., 1978b). CCK is a potent stimulator of pyloric contractions (Fisher et al., 1973; Isenberg & Csendes, 1972; Yamagishi & Debas, 1978), enhancing the slowing of gastric emptying induced by relaxation of the proximal stomach. Contraction of the pylorus and enhanced antral peristalsis lead to increased trituration. CCK therefore is probably important in the regulation of the size of particles passing through to the duodenum and, in the regulation of the pH of the food passed into the duodenum.

Endogenous opiate derivatives:

Although the endogenous opiate derivatives are found in neurones (Edin, 1980) and reduce gastric emptying, their physiological function remains unknown. Physiological stimuli causing their release also remain undefined although Shea-Donohue et al., (1981), have demonstrated that the enkephalins are unlikely to be released due to
distension of the stomach. Opiate receptors have been demonstrated in
gastric muscle cells (Bitar and Makhlouf, 1981) but such a finding
does not automatically prove a physiological role. The enkephalins
induce pyloric contraction and gastric relaxation, both of which are
abolished by the morphine antagonist naloxone (Edin, 1980). Both
diamorphine and pethidine have been demonstrated to cause a marked
delay in gastric emptying in humans (Nimmo et al., 1975).

The complete role of any of the gastro-intestinal hormones remains
unresolved. They all appear to play either a direct role in motility
or an indirect role by virtue of their actions on acid secretion.
Undoubtedly there are mechanisms that have as yet received little
attention. One of the most likely of these that will probably have a
major influence on the understanding of the activity of
gastro-intestinal musculature and hence on motility and gastric
emptying is the role that calcium plays and the effect that calcium
antagonists will have on these organs. It is known for example that
oral calcium leads to gastric hypersecretion without an increase in
serum gastrin (Holtermuller, 1974). This effect is more pronounced in
patients with duodenal ulcer (Barclay et al., 1983) leading to the
conclusion that milk may be detrimental for ulcer patients, contrary
to popular belief.

The role of prostaglandins in the gastro-intestinal tract

Prostaglandins were originally discovered in the 1930s (Kurzrok &
Lieb, 1930; Von Euler, 1934) and shown to be present in seminal fluid
by Von Euler (1966) as a result of their ability to affect the state
of contraction of smooth muscle, although at that time they were not
known as prostaglandins. Seminal fluid appears to be the only site within the body in which prostaglandins are stored rather than being produced as they are required, McGiff (1981). In other organs and tissues the prostaglandins are stored as precursors, the most ubiquitous one being arachidonic acid. Prostaglandin F whose structure was elucidated by Bergstrom & Sjovall (1960), was one of the first of the prostaglandins to be the subject of intense investigation.

The formation of prostaglandins from arachidonic acid and other 20-carbon poly-unsaturated fatty acids begins with the release of these precursors, chiefly by the action of phospholipases, (Kunze & Vogt, 1971) which release arachidonic acid from membrane phospholipids which contain large quantities of this acid. The second step is common to all cells and tissues - the formation of endoperoxides, a reaction catalysed by cyclo-oxygenase (Hamberg et al., 1974), followed by tissue-specific reactions to form active products. For example different enzymes result in prostacyclin $\text{I}_2$ ($\text{PGI}_2$) being formed in the vasculature, prostaglandin $\text{E}_2$, ($\text{PGE}_2$) in the gastro-intestinal tract, thromboxanes in platelets, etc. A few synthetic pathways are shown in Figure 1.5.

The synthesis of the prostaglandins also involves the cyclic nucleotides and as shown by Kuehl et al. (1973), the synthesis of the F series is related to activation of cyclic guanosine mono-phosphate (cGMP) and that of the E series to cyclic adenosine mono-phosphate (cAMP). There also exist mechanisms for producing one from the other. For example, Wong and co-workers, (1977) have shown that prostaglandin $\text{F}_{2\alpha}$ can be produced from $\text{PGE}_2$ by reduction of the 9-keto group of $\text{PGE}_2$, a reaction catalysed by PGE-9-keto-reductase.
Figure 1.5 The metabolism of arachidonic acid to form prostaglandins, prostacyclins and thromboxanes. Eicosatrienoic and eicosapentaenoic acids, bracketed at the top of the figure, give rise to other products that have one and three double bonds, respectively. (From McGiff, 1981)
Wong and his colleagues have also demonstrated that this particular reaction is stimulated by bradykinin. Thus, although the formation of a particular prostaglandin may be triggered by a stimulus, this production can subsequently be modified to produce a different prostaglandin with a different activity. This ability to alter the type of activity desired is probably very important in the regulatory role played by this series of natural substances.

The formation of prostacyclins in the vasculature is particularly interesting as MacIntyre et al. (1978) have shown that, due to the presence of what has become known as a plasma factor, vascular PGI₂ synthesis can be stimulated by human plasma. PGI₂ was shown by Vane and colleagues in 1976 to be a short lived metabolite of the prostaglandin endoperoxides PGG₂ and PGH₂ which inhibits platelet aggregation. However the short lived nature of PGI₂ can be overcome by its conversion to an active metabolite, 6-keto-PGE₁, which has prolonged activity.

Concentrations of most prostaglandins in the blood, particularly in arterial blood, are generally low (less than 20 pg/ml), as many, e.g. PGE₂ and PGE₁, are rapidly degraded as they pass through the lungs (Ferreira & Vane, 1967). Notable exceptions to this are prostaglandin A₂, (PGA₂) (McGiff et al., 1969), and PGI₂ (Moncada & Vane, 1977) which are not destroyed in the lungs. These latter two prostaglandins can therefore act as circulating hormones, rather than as local tissue hormones. As local hormones, most of the actions of the prostaglandins are a consequence of their local effects achieved at very low concentrations.

Prostaglandins and the related prostacyclins and thromboxanes probably all have a common purpose that is related to maintaining
homeostasis. They appear to be released in order to re-establish normal function, often through the regulation of blood flow and metabolism. For example, hypoxia triggers prostaglandin release in skeletal muscle (Messina et al., 1977). Within this context, they can be considered as agents of defence.

Prostaglandins appear to have three major roles in the stomach. These are effects on motility, cyto-protection and anti-secretory properties. The latter two are not necessarily related (Muller et al., 1981). Prostaglandins may be involved in the maintenance of tone. The criteria required to establish that prostaglandins are physiological mediators in the stomach are almost, but not unquestionably, met. Prostaglandins are found in high concentrations in the rat stomach (Bennett et al., 1967) and the human stomach can metabolise prostaglandins (Peskar & Peskar, 1976; Spenney, 1979). The stomach mucosa can also synthesise prostaglandins (Peskar et al., 1980; Konturek et al., 1979; Peskar, 1977) in both the fundal and antral mucosa (LeDuc & Needleman, 1980), although metabolism occurs more rapidly in the antrum (Spenney, 1979).

Prostaglandins are released from the rat stomach following distension (Bennett et al., 1967) and by hyperosmolarity (Knapp et al., 1978). However, they do not appear to be regulators of gastric secretions since inhibition of prostaglandin synthesis by indomethacin does not alter acid output (Tepperman et al., 1981), and pentagastrin stimulation only induces a small change in PGE₂ output (Bennett, 1976). Exogenous PGE₂ and PGI₂ do, however, reduce acid secretion in dogs and rats (Gerkens et al., 1978; Whittle et al., 1978).

Exogenous PGE₂ causes increased gastric emptying in man (Nylandeer & Mattsson, 1975) and in monkeys (Nompleggi et al., 1980). However
gastric emptying and gastric secretion after a water load were unaffected by indomethacin in monkeys at a dose that completely inhibits the conversion of arachidonic acid to prostaglandins (Nompleggi et al., 1979). This suggests that prostaglandins are not involved in the physiological control of gastric emptying. PGE₂ causes an increase in intraluminal pressure in vivo and contraction of the rat fundus preparation (Main & Whittle, 1975). Both PGE₂ and PGF₂α contract human stomach longitudinal muscle; PGF₂α also contracts circular muscle; PGE₂, however, relaxes circular muscle (Bennett & Posner, 1971). As contraction of longitudinal muscle and relaxation of circular muscle are conditions required for peristalsis, PGE₂ is most probably involved in peristalsis.

As stated earlier, the primary role of the prostaglandins and related substances is probably one of regulatory control to maintain the gastro-intestinal tract in a functional state rather than as mediators of gastric function per se.

The control of gastric emptying

Gastric emptying is controlled by all three regions of the stomach. The proximal or fundic region acts as a reservoir and exhibits slow, sustained contractions; emptying of liquids from the stomach is mainly under the influence of the proximal stomach (Wilbur and Kelly, 1973). The antrum is concerned with mixing, breaking up the food into smaller particles (trituration) and expulsion of solids; the food is forced against a closed or partly closed pylorus by the antral contractions, most of the food is forced back into the antrum and
a small quantity leaves the stomach usually only when the particles are less than 2mm in diameter, (dog) (Meyer et al., 1979), or less than 0.5mm in diameter, (human) (Mazzotta & Malagelada, 1981). That the latter is a generalization is demonstrated by the finding of a set of dentures in the jejunum of a human who died (from other causes) 13 minutes after swallowing them, (Morgan, 1945). The pylorus is the final control of gastric emptying as far as the stomach itself is concerned but, as discussed later, this organ only plays a minor role in the control of gastric emptying, its main function being to prevent duodenal reflux, (Fisher & Cohen, 1973).

Gastric motility is controlled and influenced by many factors mediated via nervous or hormonal mechanisms or by a combination of the two. Emotional states also influence gastric motility. Pain, anxiety, sadness and hostility have expression in changes in gastric motility (Davenport, 1977). The predominant nervous control of the stomach is vagal. However, this vagal innervation is very complex and can be excitatory or inhibitory on the same part of the stomach under different conditions, or be excitatory in one part of the stomach and inhibitory in another at the same time. Vagal innervation is also influenced by sympathetic tone, stomach and duodenal contents via vago-vagal and other reflexes and by hormonal influences.

Stimulation of the vagal nerve results in contractions, in particular an increase in the force and number of antral contractions (Lombardi et al., 1981), which are atropine-sensitive (Downing & Morris, 1979). In the presence of muscarinic blockade, stimulation of the vagus results in relaxation which is sensitive to ganglionic blockade (Beani et al., 1971). In the absence of muscarinic blockade but in the presence of ganglionic blockade, stimulation of the vagus also
results in relaxation which is sensitive to hyoscine (Downing & Morris, 1979). (see Figure 1.6). Therefore there appear to be atropine-sensitive excitatory fibres that involve ganglia, and inhibitory fibres, also atropine-sensitive, which either do not involve ganglia or contain muscarinic receptors on the ganglion cells. The relaxation induced in the presence of atropine is mediated via non-cholinergic, non-adrenergic nerves (Beani et al., 1971). Differing degrees of vagal stimulation result in differing responses. Low threshold cholinergic fibres enhance gastric motility, whereas high threshold fibres decrease gastric motility via non-cholinergic, non-adrenergic fibres (Martinson, 1965).

The types of neurones involved in the non-cholinergic, non-adrenergic pathways remain unknown although a number have been proposed, for example peptidergic neurones (Angel et al., 1981). Purinergic neurones have also been suggested (Burnstock et al., 1970), and in vitro work with dipyridamole; a pyrimidopyrimidine derivative which protects nucleotides from enzymic destruction (Bunag et al., 1964), and hence potentiates the effects of nucleotides (Stafford, 1966); showed that the action of both adenosine triphosphate (ATP) and stimulation of non-adrenergic inhibitory nerves in the stomach were potentiated by dipyridamole. However, in vivo stimulation of vagal inhibitory nerves to the cat stomach were not enhanced by pyridylisatogen tosylate, a specific antagonist of ATP (Heazell, 1975). The hypothesis that the non-cholinergic, non-adrenergic nerves are purinergic was therefore not supported.

Substance P, enkephalin and vasoactive polypeptide (VIP) are all present in the vagal nerves (Edin, 1980). VIP in fact meets many of the criteria to be considered as a neuro-transmitter substance. It
Figure 1.6 Illustrated are three components of the vagal nerve that influence gastric contractions. Stimulation of component 1 results in increased force and frequency of contractions that are inhibited by atropine. Stimulation of component 2 in the presence of muscarinic blockade causes a relaxation that is inhibited by ganglion blockers. Stimulation of component 3 in the presence of ganglionic blockade results in a relaxation that is inhibited by atropine acting either at postganglionic receptors or at muscarinic receptors at the ganglia.
mimics the effect of non-adrenergic, non-cholinergic vagal stimulation in the dog resulting in abolition of spontaneous action potentials and decreasing tone; it is present in the muscularis mucosa and it is released on nerve stimulation (Angel et al., 1981). Not all vagal excitatory fibres however are cholinergic. The vagus nerves supplying the pylorus in the cat contain both excitatory and inhibitory fibres, neither of which are either cholinergic or adrenergic (Edin, 1980). This is in contrast to the cholinergically innervated gastric contractions in the cat.

Afferent vagal nerves are as equally complex and even less understood than efferent nerves. Afferent vagal activation causes pyloric contraction and gastric relaxation via a vago-vagal reflex (Edin, 1980). Reflex mechanisms may also involve the sympathetic nerves, intrinsic plexuses and hormones (Davenport, 1977). Afferent impulses are initiated from the stomach probably as a result of both chemical and mechanical receptors (Iggo, 1957; Harper et al., 1959; Paintal, 1954), and probably serve as a feedback mechanism reflecting the content and distention of the stomach.

The sympathetic nervous system can induce excitation or inhibition of gastric contractions. Excitatory responses mediated via adreno-receptors have been demonstrated in a number of species including man, guinea-pig and rat, (Haffner et al., 1969; Guimaraes, 1969; Innes & Kohli, 1969), which have been shown by Bailey (1971), to be \( \alpha \)-mediated in the guinea-pig. Inhibitory responses appear to be mediated by both \( \alpha \) and \( \beta \)-receptors (Bailey, 1971; Daniel, 1966).

Dopaminergic neurones may also be involved in the contractility of the stomach. Stimulation of these neurones causes a decrease in gastric emptying. Some drugs which increase the rate of gastric
emptying are thought to act by antagonising endogenous dopamine (Pinder et al., 1976). Metoclopramide for example stimulates gastric emptying only when the latter is abnormally long or when other abnormal conditions prevail. This drug appears to act by increasing the co-ordination between stomach and duodenum (Pinder et al., 1976), in contrast to the loss of co-ordination induced by motilin. Whether dopamine acts on specific dopaminergic receptors or on adrenergic receptors has not been conclusively demonstrated (Thompson & de Carle, 1981).

Many chemicals have been proposed as having a role in the control of gastric motility. Some of these, such as VIP may be neuro-transmitters, others may not be released from neurones but released as a result of nerve impulses, pH changes, stomach distension, liquids and other food constituents and by other hormones. For example, VIP inhibits gastrin release in vivo but not in vitro and secretin inhibits gastrin release in vitro (Saffouri et al., 1981). Among those proposed as being involved in the control of gastric motility, the most important are gastrin, motilin, VIP, secretin, the enkephalins, prostaglandins, prostacyclins and cholecystokinin (CCK). Many of these hormones occur in more than one form (e.g. gastrin) and some probably describe an action that may involve a number of different substances but which are conveniently classified under one name (eg. CCK).
The role of the duodenum in controlling gastric emptying

Evidence that the duodenum influences the rate of gastric emptying, particularly to inhibit it, was demonstrated many years ago, See for example, Best & Cohnheim (1910), Cohnheim & Dreyfus (1908) and Tobler (1905). It was found that in dogs with duodenal fistulas, the chyme lost through a fistula had to be re-inserted into the intestine in order to reduce the rate of gastric emptying. It has also been known since the beginning of the century that non-irritating liquids leave the stomach rapidly compared to the time taken for irritating liquids to leave. Digestibility of food has usually been judged by the duration of its stay in the stomach.

Isotonicity is an important determinant of the rate of gastric emptying; isotonic solutions leaving the stomach faster than either hypertonic or hypotonic solutions, (Cooke, 1975; Carnot & Chassevant, 1905). Both duodenal acidification, (Cohnheim & Best, 1910), and concentrated alkaline solutions, (Spencer et al., 1916), delay gastric emptying. As with tonicity, therefore, the duodenum controls gastric emptying in order to prevent an unacceptable concentration of noxious material within its lumen.

The presence of fat in food markedly delays gastric emptying and this fact has lead to several theories concerning the method by which this inhibition of gastric emptying is achieved.

The first theory was that of a reflex closure of the pylorus. When the duodenum received material in such a concentration that the pH, osmolarity, fat content, or other constituent was too high, then a reflex closure of the pylorus was triggered. There appeared to be
some evidence for this theory, for example Marbaix (1898) and Tobler (1905), but what they were studying was inhibition of gastric emptying per se, and not the mechanism. In other words they assumed a closure of the pylorus as being the cause of the inhibition of gastric emptying which they were undoubtedly recording. However, Von Mering (1893) had suggested that the delay in gastric emptying could be due to a decreased gastric muscular activity rather than to a closure of the pylorus. In 1897 he had shown that despite a resected pylorus, which could not close, the expulsion of various stomach contents occurred at the same rates, either fast or slow, as occurred in the presence of a normal pylorus. Thus the reflex triggered from the duodenum was shown to reduce the activity of the gastric muscle, although constriction of the pyloric sphincter does appear to occur in response to appropriate duodenal stimulation under some conditions.

The second theory was simply that fat, e.g. olive oil, slowed gastric emptying by virtue of its viscosity, Moritz (1901). That high viscosity does reduce gastric emptying is beyond dispute (Prescott, 1974), but Edelmann (1906) did dispute the viscosity theory to explain the long emptying time of fats as Vaseline did not cause a similar long delay.

The first theory, concerning a reflex closure of the pylorus, had been superseded by the viscosity theory but re-emerged in the 1930s. Thomas et al. (1934) showed that the reflex acting on the gastric musculature occurred in vagotomised animals, therefore indicating mediation via the myenteric plexus. It is of particular interest to the results presented in the following chapters of this thesis that whilst acid could trigger the reflex when administered to the upper, and as far down as the middle of, the small intestine, fat could only
trigger the reflex from the upper small intestine, Best (1911). Fat however has no influence on gastric motility whilst it is present in the stomach but is only effective when placed in the upper part of the small intestine. This inhibitory effect of fat was abolished by novocaine (Best & Cohnheim, 1910).

A further theory arose from the work of Ivy & Farrell (1926) who showed that fat in the intestine inhibited the activity of a transplanted, and therefore denervated, gastric pouch. The explanation for this effect was that a hormonal mechanism was involved. Lim and his co-workers (Kosaka & Lim, 1930; Lim, 1933) have since shown that fats inhibited gastric emptying by the release of a hormone, enterogasterone. However, the hormone theory was also not the final explanation as Harris et al. (1947) showed that intravenous enterogasterone inhibited the vagally-intact stomach but not the vagally-denervated stomach. Furthermore Waddell & Wang, (1953) reported that vagotomy greatly increased the emptying of high-fat meals in patients with gastroenterostomy, thus indicating that the duodenal gastric reflex may be important in the control of gastric emptying of fats. Thus both a nervous and a hormonal component appear to be responsible for the control of gastric emptying of fats and these effects can either work independently or together.

Much of the work referred to above was done in dogs and although it is unlikely that fats act on the stomach by totally different mechanisms in different species, the relative importance of nervous v hormonal components of the reflex could differ significantly between species. Thus it may be wrong to assume that all the above results apply equally to all species. It is also possible that it is not the fat per se that triggers the reflex, but the bile acids
released by the fat. There may also be a contribution from the energy content of fats. In mixed meals of fat, carbohydrate and protein, Hunt & Stubbs (1975) showed that slowing of gastric emptying allowed the same energy content to pass into the small intestine in unit time. If more calories were present in the stomach then smaller volumes were passed into the duodenum. It was possible to predict mean half-times of gastric emptying from the energy content of meals. Although the isotonicity of solutions is important as a control of the rate of gastric emptying, whether the solution is of salt, glucose or some other solute, in the case of glucose there appears to be an additional, more specific, control depending on its concentration. Again evidence is contradictory; some suggesting that this is a vagally-mediated enterogastric reflex, others suggesting a hormonal control or, as seems most likely, a combination of the two (Quigley & Phelps, 1934). The use of dextrans, which cause inhibition of gastric emptying but do not introduce the isotonicity factor, have proved useful in helping to determine the mechanism of action and indicate, initially at least, that a nervous reflex is involved. Thomas and Crider (1936, 1939) have also shown that the inhibition of gastric emptying induced by the products of protein digestion was probably a nervous reflex that did not involve a hormonal mechanism at all.

The term enterogastric reflex was coined to describe the control of gastric motility by acid in the intestine. This reflex is greatly modified by vagotomy although a myenteric reflex also appears to be involved (Thomas et al., 1934). Other irritants, specifically 5% aqueous sodium chloride and 10% aqueous ethyl alcohol, were also found to have very similar effects to hydrochloric acid in causing an inhibition of gastric peristalsis when placed in the intestine.
Two physiological functions control gastric emptying. One is the opening and closing of the pylorus by the pyloric sphincter and the other is the alteration in the tone and activity of the stomach muscles. The former (effects on the pylorus) were initially looked upon, erroneously, as the most probable mechanism for the control of gastric emptying. It was assumed that a sphincter at the 'end' of the stomach must be there to control gastric emptying. This led to the pyloric reflex theory discussed above. Also, as acid is such an important component of normal stomach contents, it was also assumed that acid control of gastric emptying via the pylorus was the major regulatory mechanism. However, it is now well known that the acid in the stomach does not open the pyloric sphincter (eg. Carlson & Litt, 1924; McCann, 1929; McSwiney & Pyrah, 1932), nor does acid in the intestine have more than a minor effect on the pyloric sphincter, (Quigley et al., 1942). The pylorus has a minimal part to play in the control of gastric emptying, its main role being to control gastric reflux. Other factors that inhibit gastric emptying include exercise (Hellebrandt & Tepper, 1934), changes in oxygen tension (Stickney & Van Liere, 1942; Van Liere et al., 1933, 1936), environmental temperature (Sleeth & Van Liere, 1937) and emotional disturbances. (Bennett & Venables, 1920; Cannon, 1898; Quigley et al., 1943), particularly stress.

Nervous control of the gastrointestinal tract can be summarized as a pattern that moves from voluntary control at each end, (swallowing and defaecation), largely through centrally-controlled mechanisms, (oesophageal peristalsis and rectal peristalsis), to mechanisms independent of the central nervous system (gastric emptying and intestinal propulsion). The stomach, small intestine, and most of the large intestine can therefore function in the absence of centrally-
mediated nervous control. Although all these parts of the gastrointestinal tract may be influenced by autonomic nerves, their basic control is under intrinsic autonomous myenteric control systems such as the myenteric plexus. A generalisation can be made that virtually all substances entering the duodenum, that have an effect on the rate of gastric emptying, tend to decrease the rate rather than to increase it.
The history of chromones

The origin of the chromone series of drugs lies in the natural product khellin (see Figure 1.7), that occurs in the seeds of Ammi visnaga (Umbelliferae), a plant from the Middle East. Khellin was known to have vasodilatory (Bagouri, 1949) and bronchodilatory activity. The aim of the research programme begun in 1954 by Fisons was to develop the bronchodilator activity of khellin for use in the treatment of asthma and at the same time to reduce the cardiovascular side-effects. To increase solubility, the research was directed towards the 2-carboxychromones which were more soluble than the 2-methylchromones of khellin and its direct derivatives.

![Khellin](image)

Research was severely handicapped by the lack of an animal model of human asthma. Guinea-pig anaphylaxis, and other pharmacological studies, demonstrated that none of the synthesised chromones had any greater activity as bronchodilators than had khellin itself. Additionally anti-spasmogenic activity was negligible (Cox et al., 1970). The whole chromone research programme may then have floundered had it not been for the appointment of Dr. R.E.C. Altounyan as a
clinical pharmacologist. Dr. Altounyan is an allergic asthmatic and because of the very low toxicity of the chromone-2-carboxylic acids, was willing to test them on himself.

Despite their poor bronchodilator and anti-spasmogenetic activity it was found that these chemicals were able to prevent or reverse allergic bronchospasm induced by a standard antigen challenge. Activity however was of short duration until in 1963 a series of bis-chromones, (two carboxychromone molecules linked by an alkylene dioxy chain) were synthesised (Fitzmaurice & Lee, 1969). In 1965 one of these, designated FPL 670 (sodium cromoglycate, see Figure 1.8), was found to have anti-allergic activity in man that lasted for several hours (Altounyan, 1967; Pepys, et al., 1968). After further studies an extensive research programme was begun to develop FPL 670 for clinical use in asthma.

![Figure 1.8 Sodium cromoglycate](image)

FPL 670, the disodium salt of 1,3-bis (2-carboxychromon-5-epoxy)-2-hydroxypropane, disodium cromoglycate, sodium cromoglycate, was introduced into Britain in 1968 as Intal and has since been marketed world-wide as an effective and safe treatment for asthma and other allergic conditions.
Sodium cromoglycate is not an anti-inflammatory agent, a bronchodilator nor a pharmacological antagonist of mediators, nor has it any corticosteroid-like effects (Pepys, 1973). The activity of the drug is due to its ability to inhibit IgE antibody/antigen reactions by a stabilizing effect on mast cells. By this mechanism, degranulation with the consequential release of pharmacologically active mediators is inhibited (Spataro & Bosmann, 1976). How this activity occurs is not fully understood but sodium cromoglycate may stabilize the mast cell membranes by indirectly blocking the entrance of calcium ions which are essential for mediator release (Orr, 1973; Spataro & Bosmann, 1976). See Figure 1.9.

Figure 1.9 Illustrated is one of the possible mechanisms for the activity of sodium cromoglycate in asthma. The drug may indirectly block the entrance of calcium into the mast cell where it is essential for mediator release.
Although sodium cromoglycate shows marked anti-allergic activity against the 'early' antigen-induced response, it also has a long-term prophylactic activity that reduces the hyper-reactivity that normally occurs on repeated exposure to antigen. (Altounyan, 1970; Dickson, 1970). This reduction in hyper-reactivity probably occurs as a result of the drug's ability to inhibit the 'late' anaphylactic response that appears to sensitize the bronchi to anaphylactic mediators (Cockcroft et al., 1977). A second mode of action of sodium cromoglycate has also been proposed to explain its activity in both exercise-induced (Davies, 1968), and sulphur dioxide-induced (DeVries et al., 1976) bronchospasm, neither of which conditions appear to result in mediator release (Altounyan, 1979). An inhibitory effect of the drug on a reduced sympathetic tone, that is induced by exercise or sulphur dioxide, may explain the activity in these conditions. This hypothesis however, although being studied, has not been proven.

Because of the low solubility of Intal, conventional aerosol administration was found to be unsuitable as a delivery system. A novel turbo-inhaler, the Spinhaler, was therefore developed. Despite the simplicity of using the Spinhaler, patient compliance, particularly amongst children, has always been less than perfect - as it is with all other aerosol administered drugs (Eney & Goldstein, 1976). Analysis of sodium cromoglycate in urine specimens of 186 children taking Intal demonstrated that 13 % were non-compliant (i.e. were either not taking the drug at all or were not using the Spinhaler properly) and a further 16 % were either only partially compliant or whose use of the Spinhaler was poor (Morrison-Smith & Pizarro, 1972). Although these results were not considered to be unacceptable, they were less than optimal. An orally active drug with
the same or improved activity over Intal had for a long time been a desirable alternative. Therefore in 1972 the search began for a chromone or related chemical with the efficacy of Intal but with oral activity. Once again the lack of a specific animal model for human asthma hindered the early development although the percutaneous anaphylactic (PCA) response in rats proved to be an acceptable screen that gave a reasonable correlation with anti-human asthmatic activity and was used extensively during the 1970s.

In 1974 proxicromil was synthesised and found to have good anti-allergic activity in animals, particularly in the rat PCA screen, after oral administration. Extensive pharmacological and safety evaluation testing then followed with the drug being introduced into clinical trials in 1976.
The toxicity of proxicromil

Proxicromil is a chromone carboxylic acid derivative (6,7,8,9-tetrahydro-5-hydroxy-4-oxo-10-propyl-4H-1-naphtho[2,3-b]pyran-2-carboxylic acid) (see Figure 1.10), that has anti-allergic properties and is absorbed and active after oral administration.

The toxicity profile of the drug was evaluated in almost a hundred animal toxicity experiments (listed in the Appendix, Table 1). Single doses of the drug administered orally to a number of species including mice, rats, rabbits, cats, guinea-pigs and squirrel monkeys showed the drug to have a low order of acute toxicity. Where LD$_{50}$s were determined (rodents) they were approximately 2000 mg/kg orally and 100 mg/kg intravenously. Pharmacodynamic studies showed the compound to have no specific or potent effects on behaviour, on the central and autonomic nervous systems, or on the cardiovascular and respiratory systems. In all of these systems the maximum no-effect dose was greater than 50 mg/kg orally and 3 mg/kg intravenously.

![Proxicromil](image)

Figure 1.10 · Proxicromil (FPL 57787)
A range of repeated-dose studies was carried out in rats and in four non-rodent species. In these studies animals were dosed daily for up to six-months. Apart from dogs, in which hepatotoxicity was seen at doses of 60 mg/kg/day and above, the main finding in these studies was some evidence of a dose-related irritant effect upon the gastrointestinal tract at high doses. However 80 mg/kg/day was without serious effect in the majority of cynomolgus monkeys (the most susceptible species) over 180 consecutive days of treatment at this dose level. Similarly 80 mg/kg/day was very well tolerated by baboons for 180 days. Squirrel monkeys tolerated 100 mg/kg/day with few adverse effects. Generally rodents were insensitive to these actions of proxicromil.

A mild anti-coagulant effect of proxicromil was detected in vitamin K-deficient male rats. Careful evaluation of this finding led to the conclusions that it was a very mild effect in contrast to warfarin which was 5,000 to 10,0000 times more potent and that the effect had only been due to a vitamin K-deficient diet that was unknowingly being fed to the animals. There were some renal lesions (bilateral tubular ectasia) in three cynomolgus monkeys that became moribund as a result of treatment with 80 mg/kg/day of proxicromil. However it was considered that these lesions may have been secondary to the severe dehydration that occurred in these animals and the consequent effects of high concentrations of urinary constituents (including the drug and its metabolites) passing through the kidneys. No other studies showed evidence of renal toxicity.

Evaluation of the drug for its potential effects on reproduction and its potential as a teratogen was carried out in rabbits and rats. The results indicated that proxicromil had no detectable effects on
fertility, nor on development of the foetus in utero, nor on peri/post-natal development. Furthermore the drug did not adversely affect the general development of young rats in a study on endocrine function.

A range of mutagenicity tests was completed in which the drug showed no mutagenic potential even at relatively high dose levels and concentrations. A series of studies also provided evidence for the lack of effect of proxicromil on host defense mechanisms. Similarly, interaction studies with a number of drugs likely to be administered concurrently with proxicromil were negative.

Life-time carcinogenicity studies were carried out in hamsters and rats. Although there was no indication of any carcinogenic potential in the hamster study, an incidence of renal tumours occurred in the rat study that led to the conclusion that proxicromil could have been the cause. Subsequent studies showed that the activity of proxicromil in this respect was likely to be as a tumour promoter and not as a tumour inducer. The effect only occurred at very high doses that probably saturated metabolic processes and thus would not be applicable in clinical use. Nevertheless, the drug was withdrawn from clinical trials, (a decision influenced by the high incidence of gastro-intestinal side-effects in man), and plans for the imminent marketing of the drug were abandoned.

Drug kinetic studies showed that proxicromil was well absorbed after oral administration. The drug was rapidly cleared from the blood and from tissues and was excreted in the urine and faeces as unchanged compound or as metabolites. Proxicromil was extensively metabolised by hydroxylation of the alicyclic ring in man, rat, rabbit, cynomolgus monkey, baboon, and hamster. Dogs however did not readily
metabolise the drug. Experiments indicated that the rate of metabolism was a major factor in determining plasma clearance. Dogs therefore had a much lower plasma clearance than the other species listed above, which were capable of substantial metabolism, and led to the severe toxicity of the liver that was unique to the dog.

From all of these studies it was concluded that proxicromil showed a moderately low order of toxicity in acute and in repeated-dose studies and was without adverse effects upon the majority of physiological systems and functions even at high dose levels. The most likely side-effect anticipated with high doses was a degree of irritation to the gastro-intestinal tract. This was however considered unlikely to occur at clinically useful doses. It was therefore surprising to discover the high incidence of gastro-intestinal side effects that occurred when it was introduced into clinical trials.

In humans it was found that proxicromil caused gastro-intestinal side-effects in 10-15% of volunteers or patients. In a typical trial in volunteers, 1 mg/kg of proxicromil administered orally to six volunteers known to be intolerant to the drug, caused a total of 20 side-effects that were of a gastro-intestinal nature (Thomas, 1979a). The majority of these side-effects occurred 0.5 to 1.5 h after the administration of proxicromil.

The results of this trial are summarized in Table 1.2.
Table 1.2 Incidence of gastro-intestinal side-effects reported by six volunteers after taking proxicromil at 1 mg/kg orally

Half of these gastro-intestinal side-effects were of moderate or severe severity.

Data from Thomas, (1979a).

<table>
<thead>
<tr>
<th>Symptom</th>
<th>No. of volunteers reporting the symptom (out of 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nausea</td>
<td>(5 Subjects)</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>(4 Subjects)</td>
</tr>
<tr>
<td>Epigastric-pain/Heartburn</td>
<td>(4 Subjects)</td>
</tr>
<tr>
<td>Colic</td>
<td>(4 Subjects)</td>
</tr>
<tr>
<td>Flatulence</td>
<td>(2 Subjects)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>(1 Subject)</td>
</tr>
</tbody>
</table>

Such a range, severity and incidence of side-effects should have been predicted and led to the work reported in the following chapters.
CHAPTER 2

METHODS FOR STUDYING GASTRIC EMPTYING

AND THE EFFECT OF SOME CHROMONES

IN RATS AND MARMOSETS
2.1 INTRODUCTION

The chromone (6,7,8,9-tetrahydro-5-hydroxy-4-oxo-10-propyl-4H-1-naphtho[2,3-b]pyran-2-carboxylate, FPL 57787, proxicromil) has been reported to have gastro-intestinal side-effects (nausea, vomiting, abdominal pain) in some patients after oral administration of doses as low as 1 mg/kg bodyweight (Thomas 1979a, 1979b). Gastro-intestinal side-effects at these dose levels had not been predicted from animal studies. It was therefore considered that the gastro-intestinal side-effects seen in humans might be due to a functional rather than a pathological change. The time course of these effects (0.5 to 2h after dosing) supported a functional change. Gastro-intestinal motility had been studied in rats and mice as part of the conventional safety evaluation programme (see Appendix, Table 1), and oral doses of proxicromil up to 50 mg/kg had shown no effect. It was therefore considered that previous methods (i.e charcoal meal transit in rats, and faecal bolus production in mice) were insufficiently sensitive.

The first studies were therefore designed to study gastric emptying as a method of detecting functional changes induced by proxicromil in rodents. Later studies were designed to improve and simplify the techniques and to increase the predictability of the animal studies to man. A second Fisons oral chromone (FPL 52806) which had been shown to have minimal gastro-intestinal side-effects in humans was also studied. The marmoset, a species anatomically and probably functionally closer to man than rodents, was also used. From these experiments it became apparent that functional changes in the gastro-intestinal tract of animals in toxicity studies might affect the rate of absorption of the drug and thus the plasma concentrations, hence this aspect of oral dosing in toxicity studies was included.
2.2 METHODS

2.2.1 Transit of glass beads

The method consisted of administering small glass beads orally to rats and measuring the rate of transit from the stomach. The initial idea of using beads for this purpose arose from a paper by Christian and Johnson (1979) who used polystyrene beads to measure gastro-intestinal function in term embryos to assess the teratogenic potential of drugs administered to the mother. Actual techniques used in the present studies were developed from this original method.

Sprague Dawley (S/D) male rats weighing from 200 to 500g were used. Ferrets were found to be unsuitable because of both their tendency to vomit and the nature of their stomach contents. Five rats per group were necessary to show statistical significance with all but the most active doses. The administration of drug under test, or vehicle control was made six minutes before the administration of the glass beads.

Ten beads with diameters between 5.5 and 5.8 thousands of an inch were administered to each animal. The animals were killed at various times after administration of the glass beads by an intraperitoneal injection of sodium pentobarbitone, and were opened along the ventral surface, the oesophagus clamped immediately above the stomach and the stomach and small intestines dissected free. The contents of the intestines (divided into five equal parts) and the stomach were washed through a 50 thousands of an inch metal filter. Although recovery usually approached 100%, recovery from the stomach and each section of the intestines was expressed as the percentage of total recovery to account for any losses.
Studies with positive controls

**Metoclopramide**: As a positive control to increase the rate of gastric emptying, metoclopramide monohydrochloride \[N\text{-(diethylaminoethyl)-2-methoxy-4-amino-5-chlorobenzamide monohydrochloride}\] (Maxolon, Beecham Research Laboratories) at 5 mg/kg (po) was used. The animals were killed 60 minutes after administration of the beads.

**Codeine and atropine**: The first of three experiments using codeine phosphate at 1 mg/kg orally and atropine sulphate at 50 μg/kg orally (to separate groups) did not demonstrate any activity. These doses were therefore increased by five-fold. The animals were killed 90 minutes after administration of the beads.

Studies with proxicromil

A preliminary experiment indicated that proxicromil decreased the rate of gastric emptying. Proxicromil administered orally at 10 or 50 mg/kg in 0.5% methyl cellulose (M450) was then tested in male S/D rats. The animals were killed 90 minutes after the administration of beads. A further experiment was also done with proxicromil administered intravenously.

As an exception to the author carrying out the studies reported in Chapters 2 to 5, two experiments with proxicromil were done by a junior technician. The purpose of this exercise was to determine whether the method would be practical in routine use and how long a person, new to the method, would take to both learn and perform the experiment.
2.2.2 Development of a weight-method that estimates the volume of intragastric contents remaining at a specific time.

Proxicromil has been shown to be active in the studies reported in Sections 2.2.1 and 2.3.1 in inhibiting the rate of gastric emptying in rats. The following studies were initiated with the intention of using the method described in Section 2.2.1 to evaluate further the activity of the drug, to study some standard drugs and to attempt to establish a structure-activity relationship of a series of chromone compounds with their activity as inhibitors of gastric emptying. (The structure-activity studies are reported in Chapter 5).

During the autopsy of the rats treated with glass beads it was observed, subjectively, that the volume of the stomach contents appeared to be proportional to the number of glass beads remaining there, leading to the conclusion that when inhibition of gastric emptying occurred, not only were the glass beads retained, but so also were the 10 ml/kg of solution or suspension administered as the dose. Since gastric-emptying involves the removal of material from the stomach, a simple method is to measure the decline with time of the volume of the intragastric contents. This can be assessed by weighing the stomach and contents at specific times after the administration of a test meal (or water load). Therefore, with the exceptions noted in the methods, all experiments in this series of studies were carried out by measuring the stomach weights, rather than by counting beads.

The drug being tested was administered orally at a dose volume of 10 ml/kg via a rubber catheter (FG 10). All drugs were administered in 0.5% methyl cellulose (M450). Dosing of the animals was done in a random order between dose groups. Six minutes after dosing with the
drug, 20 ml/kg of tap water was administered orally. The purpose of this water was to increase the volume of the stomach in order to accentuate any inhibition or stimulation of gastric emptying.

All rats were killed by an intraperitoneal injection of sodium pentobarbitone (Sagatal 60 mg/kg, 3 ml/kg) 90 minutes after the water was administered. This interval was particularly suitable for demonstrating a decreased gastric emptying rate, as control stomachs (10 ml/kg of methyl cellulose followed six minutes later by 20 ml/kg of water) were virtually empty by this time. To demonstrate an increased emptying rate shorter time intervals were employed.

The stomach and contents were immediately weighed and the weights expressed as percentages of total bodyweights to take into account the variations in size of the animals and hence the weights of the stomach tissue per se and the variable volumes actually administered which were also based on bodyweights.

As well as studying the effect of proxicromil, a number of experiments were done to study the effect of commercially available drugs either as positive controls (metoclopramide, codeine phosphate and atropine sulphate); because they are known to have gastric side-effects (aspirin, sodium aspirin, ibuprofen and indomethacin); or because they have other gastric activity (cimetidine).

During the toxicological evaluation of proxicromil it had been noticed that gastro-intestinal damage in rats tended to occur more markedly during the first few days of oral treatment. By one month gastro-intestinal damage tended to be less. Although inhibition of gastric emptying may not be related to pathological damage, a number of studies were included to demonstrate whether inhibition of gastric emptying also decreased after multiple daily dosing.
2.2.3 Studies on gastro-intestinal function by X-ray photography

Both rats and marmosets were used in these experiments. The rats (male) were surplus to the Toxicology Teratology Breeding Unit and weighed approximately 350 g at the time of use. The marmosets (male) were surplus to the requirements of a six-month toxicity study. The animals were clinically healthy.

Oral administration of test drug was achieved either by mixing a solution (or a suspension) of the dose with a barium sulphate suspension and administering the two together, or by administering them separately by gavage. Whole-body X-ray photographs were then taken at various times after dosing (between 5 and 120 minutes) whilst the animals were briefly anaesthetised. Rats were anaesthetised with carbon dioxide and marmosets with halothane. The treatment of individual animals is shown in Table 2.1.
Table 2.1  Treatment schedule of animals and times of X-ray photography after dosing

X indicates the times after dosing at which X-ray photographs were taken.  M/C = 0.5% methyl cellulose as vehicle control.

<table>
<thead>
<tr>
<th>Species</th>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Time of X-ray (mins) after dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Rat</td>
<td>M/C</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>Rat</td>
<td>M/C</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>Rat</td>
<td>Proxicromil</td>
<td>50</td>
<td>X</td>
</tr>
<tr>
<td>Rat</td>
<td>M/C</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>Rat</td>
<td>Proxicromil</td>
<td>50</td>
<td>X</td>
</tr>
<tr>
<td>Rat</td>
<td>FPL 52791</td>
<td>50</td>
<td>X</td>
</tr>
<tr>
<td>Marmoset</td>
<td>M/C</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>Marmoset</td>
<td>FPL 52806</td>
<td>50</td>
<td>X</td>
</tr>
<tr>
<td>Marmoset</td>
<td>Proxicromil</td>
<td>50</td>
<td>X</td>
</tr>
</tbody>
</table>
2.2.4 Plasma concentrations of FPL 52806 after oral administration to marmosets

FPL 52791 is absorbed relatively slowly in rats after oral administration (Fisons internal document). Both FPL 52791 and its sodium salt, FPL 52806, (see Table 5.4 for structures) have also been shown to inhibit gastric emptying in rats (see this and Chapter 5), although the latter did not inhibit gastric emptying in marmosets. The slow absorption seen in rats may be due to this inhibition of gastric emptying which reduces the rate at which the drug enters the small intestine from where absorption can occur. If this hypothesis is correct then the lack of inhibition of gastric emptying in marmosets should lead to a more rapid rise in plasma concentration in this species compared to the rat. This study was designed to determine plasma concentrations of FPL 52806 at various times after an oral dose of the drug.

Two male marmosets (Calithrix jacchus) were used. Cannulae were inserted into a caudal vein of each animal and a blood sample (approximately 0.3 ml) was taken from each animal prior to dosing. 4.4 mg of \(^{14}\)C-FPL 52806 of specific activity 9.0 \(\mu\)Ci/mg was mixed with non-radiolabelled FPL 52806 to give a total concentration of FPL 52806 of 2.5 mg/ml.

The animals were orally dosed by gavage with this mixture at a dose volume of 20 ml/kg to give a dose of 50 mg/kg. Serial blood samples (each of 0.3 ml) were taken from each animal at various times after dosing (see Results). Plasma was prepared by centrifugation in a Gelman Hawkley haematocrit centrifuge. The radioactivity in 10 \(\mu\)l aliquots of each plasma sample was determined by liquid scintillation spectrometry in 5 ml of Fisofluor "mpc" scintillator, and the results expressed in equivalents of parent drug.
2.3 RESULTS

2.3.1 Transit of glass beads

Studies with positive controls

Metoclopramide

Metaclopramide at 5 mg/kg orally (approximately five times clinically effective doses) increased the rate of gastric emptying, see Table 2.2. The lack of variability was good. The drug also increased intestinal transit for up to the third fifth of the small intestine.

Codeine and atropine

Both codeine and atropine induced statistically significant decreases in the rate of gastric emptying, see Table 2.3.

The method was therefore shown to have demonstrated significant responses to drug induced increases and decreases in the rate of gastric emptying.

Studies with proxicromil

The first two of these experiments were carried out to determine whether the method was suitable for routine use as a toxicology screen by an otherwise untrained technician. A satisfactory 98% recovery of beads on the second attempt by an otherwise untrained person demonstrated the practicability of the method and the minimal training required to use the method routinely.

Proxicromil showed a dose-related decrease in the rate of gastric emptying, see Table 2.4. This decrease was very significant \((p < 0.01)\) at 50 mg/kg.
Table 2.2  Mean percent recoveries of glass beads from the stomach and from each 20% length of the small intestine 60 minutes after 5 mg/kg of metoclopramide

Means of 5 animals per group

Significant reductions ***, or increases ###, in glass beads at the stated sites after metoclopramide compared to control values (p < 0.001).

Five equal lengths of the small intestine shown as percentages of the total length with zero commencing at the gastric end.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stomach</th>
<th>0-20</th>
<th>21-40</th>
<th>41-60</th>
<th>61-80</th>
<th>81-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>58.4</td>
<td>6.0</td>
<td>31.0</td>
<td>4.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Metoclopramide</td>
<td>0***</td>
<td>0</td>
<td>0***</td>
<td>100###</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 2.3  Mean percent recoveries of glass beads from the stomach and from each 20% of the small intestine 90 minutes after 5 mg/kg of codeine or 250 µg/kg of atropine

These doses were approximately 2 to 10 times clinical doses.

Means of 3 animals per group

* Significant increases in glass beads after codeine and atropine compared to control values (p < 0.05).

Five equal lengths of the small intestine shown as percentages of the total length with zero commencing at the gastric end.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stomach</th>
<th>0-20</th>
<th>21-40</th>
<th>41-60</th>
<th>61-80</th>
<th>81-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.3</td>
<td>6.7</td>
<td>10.0</td>
<td>50.0</td>
<td>20.0</td>
<td>0</td>
</tr>
<tr>
<td>Codeine</td>
<td>86.7*</td>
<td>0</td>
<td>10.0</td>
<td>0</td>
<td>3.3</td>
<td>0</td>
</tr>
<tr>
<td>Atropine</td>
<td>63.3*</td>
<td>3.3</td>
<td>23.3</td>
<td>3.3</td>
<td>6.7</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 2.4  Mean percent recoveries of glass beads from the stomach and from each 20% length of the small intestine after oral doses (po) of proxicromil at 10 or 50 mg/kg and intravenously (iv) at 20 mg/kg

Means of 3 animals per group

Significant increase in glass beads after proxicromil compared to control values ** (p < 0.01)

Significant decrease in glass beads after proxicromil compared to control values *** (p < 0.001).

Five equal lengths of the small intestine shown as percentages of the total length with zero commencing at the gastric end.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stomach (%)</th>
<th>Small intestine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-20</td>
</tr>
<tr>
<td>Control (po)</td>
<td>28.3</td>
<td>11.0</td>
</tr>
<tr>
<td>Proxicromil (10 mg/kg)(po)</td>
<td>80.0</td>
<td>3.3</td>
</tr>
<tr>
<td>Proxicromil (50 mg/kg)(po)</td>
<td>100.0**</td>
<td>0</td>
</tr>
<tr>
<td>Control (iv)</td>
<td>33.3</td>
<td>0</td>
</tr>
<tr>
<td>Proxicromil (20 mg/kg)(iv)</td>
<td>100.0**</td>
<td>0</td>
</tr>
</tbody>
</table>
2.3.2 Development of a weight-method that estimates the volume of intragastric contents remaining at a specific time.

Studies with proxicromil

Dose/Response studies with proxicromil

A linear response to proxicromil was obtained when the increase in stomach weights of proxicromil-treated animals compared to control animals was plotted against log concentration of proxicromil. The results are tabulated in Table 2.5 and shown graphically in Figure 2.1.

The effect of pre-treatment with proxicromil on subsequent inhibition of gastric emptying induced by the drug

It was shown that proxicromil could induce tachyphylaxis in most animals after only one day of pre-treatment with the drug. However some animals did not exhibit this phenomenon with the result that statistically significant tachyphylaxis did not always occur. After a minimum pre-treatment period of 21 consecutive days however there was a well established tachyphylaxis. The variability of response after less than 21 days pre-treatment was too great for this effect to be useful in aiding studies on the mode of action of proxicromil.

A second chromone, FPL 52806, was found to be at least as active as proxicromil in inhibiting gastric emptying in rats. Table 2.6 shows the comparative activity of these two drugs.
Table 2.5  Group mean stomach weights as percent of bodyweights

after proxicromal administered orally

Means of 5 animals per group (10 control animals) with standard errors indicated.

*** Significant increases in stomach weights compared to control values after proxicromil (p < 0.001).

N/A = Not applicable

<table>
<thead>
<tr>
<th>Proxicromil (mg/kg)</th>
<th>Stomach Weights as % Bodyweights</th>
<th>% increase v control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.16 ± 0.09</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>1.41 ± 0.21</td>
<td>22</td>
</tr>
<tr>
<td>10</td>
<td>2.20 ± 0.26</td>
<td>90 ***</td>
</tr>
<tr>
<td>50</td>
<td>2.98 ± 0.22</td>
<td>157 ***</td>
</tr>
</tbody>
</table>
Figure 2.1  Dose response relationship of proxicromil on the rate of gastric emptying in rats

Means of five animals per group with standard errors indicated
Proxicromil administered orally 90 minutes before the animals were killed and the stomachs weighed.
Data from Table 2.5
Table 2.6  Comparative activity of proxicromil and FPL 52806 in their ability to inhibit gastric emptying

Group mean stomach weights with standard errors indicated.

*** Significant reductions in gastric emptying compared with control values (p < 0.001), 90 minutes after dosing.

<table>
<thead>
<tr>
<th>Treatment (50 mg/kg)</th>
<th>Stomach Weights as % Bodyweights</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.06 ± 0.36</td>
</tr>
<tr>
<td>Proxicromil</td>
<td>4.35 ± 0.41 ***</td>
</tr>
<tr>
<td>FPL 52806</td>
<td>4.77 ± 0.39 ***</td>
</tr>
</tbody>
</table>
Expressed as a percentage of the activity of proxicromil, FPL 52806 had an inhibitory activity on gastric emptying of 111%.

Because of this very marked activity of FPL 52806, studies were done to test the hypothesis that the two drugs would cause cross-tachyphylaxis, as proxicromil had been shown to inhibit its own activity after 21 days of dosing.

FPL 52806 itself, like proxicromil, was tachyphylactic (44% reduction in activity after pre-treatment with FPL52806, \( p < 0.01 \)). However, although pre-treatment with proxicromil did not cause cross-inhibition of FPL 52806 to a significant extent \( (p > 0.05) \), pre-treatment with FPL 52806 did cause a significant reduction in the activity of proxicromil (34% \( p < 0.05 \)).

As confirmation of the study mentioned earlier FPL 52806 was shown to be at least as active as proxicromil (106% of the activity of proxicromil). Proxicromil itself induced a large inhibition of gastric emptying. It was also demonstrated that pre-treatment with proxicromil for 32 days did not influence the control response to methyl cellulose thus demonstrating that no permanent change to the control of gastric emptying had been induced by the drug.
Studies with positive controls

Atropine sulphate

An experiment designed to demonstrate whether recovery of glass beads and measurement of stomach weights resulted in the same conclusion was done using atropine sulphate as the gastric inhibitor, see Table 2.7.

Assessing the results by measurement of stomach weights revealed a 30% increase over control values and by bead retention a 45% increase over controls. Thus both methods demonstrated similar levels of activity with the bead retention method being slightly more sensitive.

Dose/response studies with codeine phosphate

A linear response to codeine was obtained when the increase in stomach weights of codeine-treated animals compared to control animals was plotted against log concentration of codeine. The results are tabulated in Table 2.8 and shown graphically in Figure 2.2. Codeine phosphate was shown to have approximately the same degree of activity as proxicromil.
Table 2.7  Group mean stomach weights as percent of bodyweights and percent recovery of glass beads after atropine sulphate administered orally at 500 µg/kg

Means of 5 animals per group with standard errors indicated. Animals killed, glass beads counted and stomachs weighed 90 minutes after administration of atropine sulphate

a) Absolute values

<table>
<thead>
<tr>
<th>Atropine Sulphate (µg/kg)</th>
<th>% of recovered beads present in Stomach</th>
<th>Stomach Weights as Percent of Bodyweights</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>62</td>
<td>2.22 ± 0.32</td>
</tr>
<tr>
<td>500</td>
<td>90</td>
<td>2.88 ± 0.34</td>
</tr>
</tbody>
</table>

b) Percentage increases over control values

<table>
<thead>
<tr>
<th>Atropine Sulphate (µg/kg)</th>
<th>recovery as % of control values</th>
<th>Stomach Weights as % of control values</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>45</td>
<td>30</td>
</tr>
<tr>
<td>p value</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>
Table 2.8  Group mean stomach weights as percent of bodyweights after codeine phosphate administered orally

Means of 5 animals per group (10 control animals) with standard errors indicated.

Stomachs weighed 90 minutes after administration of codeine phosphate.

Significant increases in stomach weights compared to control values after codeine phosphate * (p < 0.05), *** (p < 0.001).

N/A = Not applicable

<table>
<thead>
<tr>
<th>Codeine PO (mg/kg)</th>
<th>Stomach Weights as % Bodyweights</th>
<th>% increase v control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.09 ± 0.17</td>
<td>N/A</td>
</tr>
<tr>
<td>1</td>
<td>1.98 ± 0.13</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>2.88 ± 0.33</td>
<td>38 *</td>
</tr>
<tr>
<td>25</td>
<td>4.10 ± 0.19</td>
<td>96 ***</td>
</tr>
</tbody>
</table>
Figure 2.2  Dose response relationship of codeine phosphate on the rate of gastric emptying in rats

Means of five animals per group with standard errors indicated
Stomach weighed 90 minutes after the oral administration of codeine phosphate
Data from Table 2.8
The effect of metoclopramide on the gastric inhibitory activity of proxicromil

The purpose of this experiment was to determine whether metoclopramide inhibited the decrease in gastric emptying induced by proxicromil. Proxicromil alone induced a 148% increase in stomach weights compared to controls (p < 0.001). Although proxicromil with metoclopramide still induced a significant increase in weight over control values (84% p < 0.001) this effect was significantly less than with proxicromil alone (p < 0.05). The 50 mg/kg dose of proxicromil used was probably a maximally effective dose (as very little fluid had left the stomachs of animals receiving this dose).

The control values from this study were also used to assess whether the stomach weight data was parametric, in order to determine the most applicable method of statistically analysing the data from both this and other experiments involving rat stomach weights. A test for non-normality showed clearly that the data was not non-parametric (p > 0.05). Therefore stomach weights are analysed by parametric methods. At 25 mg/kg metoclopramide significantly inhibited the gastric effect of proxicromil. The relatively poor activity of metoclopramide in this series of experiments, see Table 2.9, was probably due to the ninety minute period between the administration of the drug and weighing of the stomachs. It was necessary to use this long period in order to demonstrate the inhibitory activity of proxicromil.
Table 2.9  The effect of metoclopramide on gastric emptying at various times after dosing

Group mean stomach weights (5 animals per group) with standard errors indicated.
Time intervals between dosing with metoclopramide orally and weighing the stomachs are shown in the table.

* No significant effect due to metoclopramide (p > 0.05).
** Significant increase in the rate of gastric emptying induced by metoclopramide (p < 0.05).

Maximum activity of metoclopramide in increasing the rate of gastric emptying was shown to be 60 minutes after administration.

<table>
<thead>
<tr>
<th>Dose Group</th>
<th>Time Interval (min)</th>
<th>Metoclopramide (mg/kg)</th>
<th>Stomach Weights as % Bodyweights</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>0</td>
<td>1.28 ± 0.14</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>10</td>
<td>1.56 ± 0.10</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>0</td>
<td>1.34 ± 0.25</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>10</td>
<td>1.06 ± 0.05</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>0</td>
<td>1.64 ± 0.13</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>10</td>
<td>1.10 ± 0.15</td>
</tr>
<tr>
<td>7</td>
<td>90</td>
<td>0</td>
<td>1.02 ± 0.08</td>
</tr>
<tr>
<td>8</td>
<td>90</td>
<td>10</td>
<td>1.22 ± 0.07</td>
</tr>
</tbody>
</table>
Drugs with known effects on the gastro-intestinal tract

Proxicromil as a positive control induced a 95% inhibition of gastric emptying compared to control values. As shown in Table 2.10, neither aspirin, sodium aspirin, indomethacin, ibuprofen nor cimetidine had any effect on the rate of gastric emptying.

Table 2.10 The effects of aspirin, sodium aspirin, indomethacin, ibuprofen and cimetidine on the rate of gastric emptying compared to the activity of proxicromil

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stomach Weights as % Bodyweights</th>
<th>% Change from Control values</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Control)</td>
<td>2.77 ± 0.32</td>
<td>N/A</td>
</tr>
<tr>
<td>Proxicromil</td>
<td>5.39 ± 0.55</td>
<td>95 ***</td>
</tr>
<tr>
<td>Aspirin</td>
<td>2.76 ± 0.08</td>
<td>0 *</td>
</tr>
<tr>
<td>Na Aspirin</td>
<td>2.56 ± 0.31</td>
<td>0 *</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>2.59 ± 0.63</td>
<td>0 *</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>2.21 ± 0.41</td>
<td>0 *</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>2.44 ± 0.24</td>
<td>0 *</td>
</tr>
</tbody>
</table>

# No significant activity compared to control values (p > 0.05).
*** Significant inhibition of gastric emptying (p < 0.001).
N/A = Not applicable
2.3.3 Studies on gastro-intestinal function by X-ray photography

Within 5 minutes of the oral administration of a barium sulphate suspension to rats, the contrast medium had progressed 20 to 30 cm along the small intestine (See Figures 2.3 & 2.5). When proxicromil or FPL 52791 (the sodium salt of FPL 52806) were administered at the same time as the barium sulphate, the contrast medium had again progressed 20 to 30 cm along the intestine (See Figures 2.4 & 2.6). However, it was noticeable in these animals, particularly the proxicromil treated animals (See Figure 2.4), that the contrast medium did not extend back as far as the stomach, ie. there was a break in contrast medium of approximately 5 to 10 cm, between the stomach and the first section of the intestine. Thus gastric emptying had initially taken place but had then ceased although intestinal propulsion had continued. By two hours virtually all of the contrast medium had left the stomach and upper intestines of control animals (See Figure 2.7) whereas the stomachs of animals treated with either proxicromil or FPL 52791 still contained appreciable quantities of contrast medium (See Figure 2.8).

In marmosets the contrast medium had progressed 30 cm or more along the intestine by 30 mins (See Figure 2.9). By two hours the stomach was almost empty of contrast medium which at that time was found predominantly in the colon. Ten minutes after an oral dose of FPL 52806 the contrast medium had progressed approximately 20 to 30 cm along the intestine with further progression after 30 minutes. (See Figure 2.10). By two hours very little remained in the stomach, the majority appearing in the colon, as in the controls. Ten minutes after dosing with proxicromil however, no appreciable quantity of contrast medium had left the stomach and this picture remained the same for 30 minutes. (See Figure 2.11).
Figure 2.3  X-Ray photograph taken 5 min after methyl cellulose and barium sulphate administration to a rat.

Figure 2.4  X-Ray photograph taken 5 min after 50 mg/kg of proxicromil and barium sulphate administration to a rat.

Figures 2.3 and 2.4  Note the distance the contrast medium has moved within 5 minutes and the absence of contrast medium in the proximal duodenum (between the arrows) in the proxicromil treated animal.
Figure 2.5  X-Ray photograph taken 5 min after methyl cellulose and barium sulphate administration to a rat.

Figure 2.6  X-Ray photograph taken 5 min after 50 mg/kg of FPL 52791 and barium sulphate administration to a rat.

Figures 2.5 and 2.6  Note the large amount of contrast medium that has left the control stomach and the relative absence of medium in the intestine of the FPL 52791 treated animal.
Figure 2.7 X-Ray photograph taken 2 hours after methyl cellulose and barium sulphate administration to a rat.

Figure 2.8 X-Ray photograph taken 2 hours after 50 mg/kg of FPL 52791 and barium sulphate administration to a rat.

Figures 2.7 and 2.8 Note the complete absence of contrast medium in the control stomach and the large amount still remaining in the FPL 52791 treated stomach.
Figures 2.9, 2.10 and 2.11. Note the progression of the contrast medium along the intestines of the control and FPL 52806 treated animals but the absence of any gastric emptying in the proxicromil treated animal.
2.3.4 Plasma concentrations of FPL 52806 after oral administration to marmosets

Specific Activity of FPL 52806 = 0.66 μCi per mg

Dose Concentration for 10 μl dose solution = 2.5 mg per ml of FPL 52806

Therefore 1 μg of FPL 52806 = 806 dpm

Plasma concentrations of FPL 52806 are shown in Table 2.11 and graphically in Figure 2.12 along with previously obtained plasma data from rats.

Table 2.11. Plasma concentrations of FPL 52806 in marmosets

The data below illustrate the rapid absorption of FPL 52806 in marmosets and a relatively fast decline in plasma concentrations.

* Pre-dose sample  ** dpm \(\frac{μl/l}{806}\) (since 1 μg = 806 dpm)

<table>
<thead>
<tr>
<th>Animal No</th>
<th>Sample Time (h:min)</th>
<th>FPL 52806 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0* 0:10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0:20</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>0:30</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>1:00</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>1:30</td>
<td>24.5</td>
</tr>
<tr>
<td></td>
<td>2:00</td>
<td>13.2</td>
</tr>
<tr>
<td></td>
<td>3:00</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>4:00</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>5:00</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>1:00</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>1:30</td>
<td>11.1</td>
</tr>
<tr>
<td></td>
<td>2:00</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>3:00</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>4:00</td>
<td>No Sample</td>
</tr>
<tr>
<td></td>
<td>5:00</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Figure 2.12 Plasma concentrations of radioactivity after oral administration of $^{14}$C FPL 52806 to rats and marmosets

- Marmoset No. 1 after 50 mg/Kg
- Marmoset No. 2 after 50 mg/Kg
- *Mean of three rats after 100 mg/Kg

* Rat data from Fisons internal report (Department of Metabolic Studies)
A method for demonstrating inhibition of gastric emptying in vivo was developed. The method of measuring gastric emptying using glass beads was superceded by an easier and quicker method that involved only weighing the stomach and its contents; with three animals per group and five at low doses, statistical significance was achieved.

Proxicromil had a marked effect on the rate of gastric emptying, at doses as low as 10 mg/kg orally, or 20 mg/kg intravenously. The degree of inhibition after intravenous dosing was low, which indicates that oral doses probably do not depend on absorption to exert their effect. The dose of 10 mg/kg orally is approximately ten times the clinical dose that induced nausea and other gastrointestinal side-effects in man. Proxicromil also induced a linear log-dose response on the rate of gastric emptying. The reproducibility of the method was demonstrated with proxicromil which was used as a positive control in some experiments.

The rat in vivo method also detected changes in gastric emptying induced by known drugs, e.g. codeine (decrease) and metoclopramide (increase). The period between administration of the drug and autopsy was varied depending on the activity, e.g. for a drug decreasing gastric emptying, 90 minutes allowed gastric emptying to occur in control animals thus demonstrating a decreased rate, whereas for a drug increasing the rate of gastric emptying, 30 to 45 minutes would be more appropriate as control stomachs would then still contain most of their contents and an increased rate could be readily seen. For a drug of unknown activity, 60 minutes is probably the optimal time.
A degree of tolerance was demonstrated with proxicromil which confirms results obtained in humans that on repeated dosing the gastric side-effects (mainly nausea) tend to decrease in incidence. Similarly another chromone, FPL 52806, which was shown to be at least as active as proxicromil in decreasing the rate of gastric emptying in rats, also showed some tolerance. Both drugs also induced some cross-tolerance. The development of tolerance however was too inconsistent to be of value in determining the mode of action of either drug but did indicate the possibility of the two drugs having a common mode of action in rats.

In an attempt to visualise gastric contractions and the effect of proxicromil on them, rats were dosed orally with barium sulphate and then X-ray photographs taken. Gastric emptying and its inhibition were well demonstrated. The method confirmed earlier results that proxicromil inhibited gastric emptying in rats.

These X-ray studies indicated that the contrast medium, and with it proxicromil, appeared to have left the stomach in a small quantity before inhibition of gastric emptying had taken place. Propulsion of intestinal contents had continued, leaving a 'gap' in contrast medium in the proximal duodenum. This finding supported the belief (from studies reported in Chapter 3) that the drug acted via the duodenum.

Both proxicromil and FPL 52791 (the acid of FPL 52806) have been administered orally to humans and both are very active as inhibitors of gastric emptying in rats. However proxicromil has been reported as causing significant gastro-intestinal discomfort in humans whilst FPL 52791 has not. This is therefore at variance with the results seen in rats. In the marmoset X-ray studies it was notable that although proxicromil caused inhibition, FPL 52806 did not. (The loss
of proxicromil between 0.5 hours and 32 hours after dosing due to vomiting is to be expected when gastric emptying is almost totally abolished.) Thus the marmoset appeared to respond to these two drugs in a manner similar to man.

The rat model therefore appears to be less specific and less predictable for man than does the marmoset. However, continued use of the rat is justified as a primary screen because although it may demonstrate false positive results it is unlikely to give false negatives - an important requirement of any safety evaluation study. Any candidate drug showing a positive result in rats can then be examined in more detail in marmosets.

Plasma concentrations after orally administered FPL 52791 were already available from previous work carried out by Fisons Metabolic Studies Department. Because of the marked difference in effects on gastric emptying between marmosets and rats with this drug, the plasma concentrations were studied after oral dosing in marmosets. The slow absorption of FPL 52791 in rats resulting in low plasma concentrations (shown in Figure 2.12) could have been due to the inhibition of gastric emptying.

The present study suggests that this was the case since plasma concentrations after oral administration to marmosets reached a peak within one hour of dosing with near-maximum concentrations at the first sample time of ten minutes. Thus the lack of an inhibitory activity on gastric emptying in the marmoset allowed the drug to leave the stomach rapidly and hence be absorbed from the duodenum rapidly. The relevance of toxicity studies with this drug in rodents for prediction to man where inhibition of gastric emptying does not appear to occur may therefore be reduced. This point is discussed further in Chapter 6.
In conclusion, these studies have demonstrated the development of simple practical primary and secondary screens for evaluating the potential of a new drug to cause some gastro-intestinal symptoms in man as a result of inhibition of gastric emptying.

Some other facets arising from an inhibition of gastric emptying have been seen during these studies and are explored and discussed further in the following chapters.

Proxicromil has been shown to be a potent inhibitor of gastric emptying in both rodents and primates. Considering the nature of the side-effects induced by this drug in humans, the inhibition of gastric emptying is almost certainly the cause of the high incidence of gastro-intestinal discomfort that occurred in clinical trials.
CHAPTER 3

THE MECHANISM OF ACTION

OF PROXICROMIL-INDUCED

INHIBITION OF GASTRIC EMPTYING
3.1 INTRODUCTION

Experiments described in Chapter 2 have shown that proxicromil reduced the rate of gastric emptying in rats in vivo. To evaluate this effect of the drug it was desirable to demonstrate decreased gastric activity following the addition of the drug to an in vitro stomach preparation.

The nature of the mechanism of the gastro-intestinal effect of proxicromil was completely unknown when these experiments were initiated. The first priority was therefore to test the hypothesis that the drug had a direct effect somewhere on the musculature of the stomach, either by causing a long-lasting relaxation of the gastric muscle, a sustained contraction, or possibly an effect on the pyloric sphincter causing it to remain closed for long periods.

The first three of the experiments described in this chapter were therefore designed to demonstrate any such activity of the drug. As these studies failed to demonstrate a direct activity of the drug on the stomach, an in situ preparation was subsequently developed which was intermediate between the conscious in vivo screen described in Chapter 2 and the in vitro screen described in the early sections of this chapter.

Following the successful demonstration of an inhibitory activity of proxicromil in this in situ preparation, further studies were done to characterize the mode, site of action and nature of the activity of the drug.
3.2 METHODS

3.2.1 An organ bath in vitro method and the effect of proxicromil on stomach contractions

The medium used throughout these experiments was McEwen's solution (McEwen, 1956) gassed continuously with 95% O₂/5% CO₂ and maintained at 35 ± 1°C. Organ bath washing was by overflow. Animals were killed by cervical dislocation. The conditions pertaining to the in vivo stomach were reproduced as far as possible in vitro. To this end, two conditions were met; the stomach itself was not damaged by inserting cannulae through the wall, and the drugs tested were used in the same concentrations as had been used in vivo. Additionally, to ensure that only the contractility of the stomach was recorded, the activity of the pyloric sphincter was not involved in the preparation.

Initial experiments were used to determine the optimal conditions, procedures, and the design of the apparatus. The rats were killed and the abdomen opened so that the gastro-intestinal tract was exposed. Catheter Y was inserted through the oesophogeal junction until it lay 1 cm inside the stomach (see Figure 3.1). Catheter X was passed through the pyloric sphincter until it lay 1 cm inside the stomach. Contractions of the stomach were recorded using a P23BB pressure transducer recording on a Devices MX2 multi-channel recorder. The stomach was then distended via Catheter Y with 20 ml/kg of tap-water. After approximately 10 to 20 minutes when regular contractions had been obtained the stomach was washed and re-filled with 0.5% methyl cellulose at 20 ml/kg. The stomach was allowed to re-establish regular contractions before being washed and re-filled with either proxicromil, atropine sulphate, or with methyl cellulose solutions,
each at 20 ml/kg. Proxicromil was used at a concentration to give the equivalent of an \textit{in vivo} dose of 50 mg/kg and atropine sulphate to give the equivalent of an \textit{in vivo} dose of 1 mg/kg. Both of these doses of drugs had caused marked inhibition of gastric emptying \textit{in vivo}.

3.2.2 The effect of proxicromil on isolated fundic strip preparations

Since proxicromil was shown in the previous experiments (see Results Section 3.2.1) to have no effect on the contractions of the rat whole stomach preparation \textit{in vitro}, this study was designed to determine whether or not the drug had any inhibitory effect on the rat fundic strip. As proxicromil had failed to inhibit the contractions of the whole isolated stomach it was considered unlikely to have an effect on isolated muscle strips. However, this was an assumption and needed to be examined in order to eliminate it as a possible mode of action of the drug.

The fundic strip preparation was as described by Vane (1957). This consisted of dissecting the fundic portion of the stomach from a freshly killed rat. The fundus was cut open to form a flat sheet and then cut so as to produce a long strip of muscle. The muscle was suspended in an organ bath containing Kreb's solution warmed to 37\degree C and gassed with 95% oxygen/5% carbon dioxide. The preparations were washed by overflow and attached to an isometric strain-gauge transducer under a base-line tension of 1 g. Solutions or suspensions were added to the Kreb's solution bathing the tissue, and responses recorded via the transducer on an MX2 Devices recorder.
Changes in intraluminal stomach pressures were recorded continuously after initial distension with 0.5% methyl cellulose. Test drugs were added to the lumen of the stomach at 20 ml/kg of pre-death bodyweight. Recordings of stomach contractions were made via a P23BB pressure transducer and a Devices MX2 multi-channel pen recorder.
3.2.3  An in vitro preparation to demonstrate pyloric sphincter function

The previous studies had shown that proxicromil had no direct inhibitory activity on the isolated rat stomach despite a marked activity in vivo. The possibility that the drug exerted its activity by causing constriction of the pyloric sphincter was examined in the studies reported in this section. The method was similar to that described in Section 3.2.1 except that the stomach was left open at the pylorus with 2 to 3 cm of duodenum left attached. The stomach was bathed in McEWen's solution. The organ bath was maintained at 35 ± 1°C and was washed by overflow. The rats were killed and the stomachs prepared as described in Section 3.2.1. A dual catheter was inserted through the oesophageal junction until it lay 1 cm inside the stomach. One of the catheters was connected to a Statham P23BB pressure transducer recording on a Devices MX2 Recorder and the second catheter to a peristaltic pump.

Methyl cellulose (0.5% aqueous) as control, or a suspension or solution of the drug under test was pumped at various speeds (between 1 and 3 ml/min) into the stomach. This resulted in a build-up of pressure inside the stomach as long as the pyloric sphincter remained closed. When the sphincter opened as a result of the increased pressure, the fluid was forced out with a consequential loss of pressure. The pressure changes were monitored and the maximum pressure reached inside the stomach before the pyloric sphincter opened were recorded.
3.2.4. An in situ stomach preparation to demonstrate functional parameters and the effect of proxicromil administered into the stomach or duodenum

This study was designed to test the hypothesis that proxicromil acted indirectly on the stomach by a mechanism that was not present in vitro. The rats were anaesthetised with sodium pentobarbitone administered intravenously through an in-dwelling catheter in the tail vein. More anaesthetic was administered to maintain the level of anaesthesia during the experiments. The anaesthetised rats were opened along the midline and the stomach and the first few centimetres of the duodenum were lifted to the surface. For intraduodenal administration one short cannula was placed through an incision cut in the duodenum approximately 1 cm from the stomach. A dual cannula was inserted through the same incision in the duodenum but passed in the opposite direction, ie. into the stomach. The oesophagus was then ligated immediately distal to the stomach to prevent regurgitation with a consequential loss in stomach pressure.

To initiate stomach contractions, 0.5% aqueous methyl cellulose was administered to the stomach at 10 ml/kg. For administration of drug or vehicle into the stomach or into the duodenum a dose volume of 1.25 ml/kg was used. As described below, most animals were dosed with either proxicromil or with control methyl cellulose solution. Some animals however received proxicromil after methyl cellulose, thus serving as their own controls.
3.2.5 Dose-response relationship of proxicromil and the inhibition of gastric emptying in situ

Proxicromil was administered intraduodenally to rats prepared with stomach and duodenal cannulae as described previously in Section 3.2.4. Pressure changes occurring in the stomach were recorded. The anaesthetised rats were allowed to develop spontaneous stomach pressure changes after the administration of 0.5% methyl cellulose into the stomach. Proxicromil was then administered at 1 ml/kg into the duodenum at doses of 1 to 16 mg/kg. After each inhibiting dose, the stomachs were allowed to re-establish contractions before a further dose was administered.
3.2.6 Investigation of the site of action of proxicromil in the gastro-intestinal tract

The previous studies described in Section 3.2.4 (with results in Section 3.3.4) demonstrated that proxicromil exerted its inhibitory action on gastric emptying by an effect on the small intestine and not by an effect on the stomach itself. This inhibition of gastro-intestinal activity was demonstrated by administering the drug into the first 2 to 3 cm of the proximal duodenum. This study was designed to determine how far down the intestine this activity of the drug occurred.

The rats were prepared as described in Section 3.2.4. In those earlier studies the duodenal cannula was inserted as close to the stomach as was practical. During the present study it was found that when proxicromil was administered 80 or more centimetres from the stomach the drug was inactive. Therefore animals in this study had the duodenal cannula inserted at different points between 1 and 80 cm from the stomach in order to find where the inhibitory activity of the drug ceased.

In order to keep any disturbance of the intestines to a minimum, the position of the duodenal cannula was estimated upon initial insertion. After each animal had been treated with proxicromil and the response recorded, the animal was killed with an over-dose of sodium pentobarbitone (intravenously). The intestines were separated from the mesenteries and the actual distance of the cannula from the stomach measured. This procedure necessarily resulted in non-standard distances between the stomach and cannula insertion being used, as shown in the results.
3.2.7 Effect of FPL 57787KA on stomach pressure changes in situ

Proxicromil (FPL 57787), the relatively insoluble acid, was used in all the previously described experiments to inhibit gastric emptying; the drug was administered as a solid suspended in 0.5% methylcellulose. The possibility that its action depended upon the insoluble particulate drug was investigated in this study by using the soluble sodium salt, FPL 57787KA.

The rats were prepared as described in Section 3.2.4. The abdomen of each of the anaesthetised rats were opened and a dual cannula inserted into the stomach. A second cannula was inserted into the duodenum. FPL 57787KA, the soluble sodium salt of proxicromil, was dissolved in saline at 20 mg/ml (pH 7.0) and administered into the duodenum at 20 mg/kg. Stomach pressure changes were recorded throughout the experiment.
3.3 RESULTS

3.3.1 An organ bath in vitro method and the effect of proxicromil on stomach contractions

Spontaneous pressure increases of 5 to 10 mm of water pressure usually occurred, although increases above 20 mm of water were occasionally seen. A number of patterns (or type of response) were apparent. The most consistent spontaneous pressure increase (referred to here as a contraction) had a frequency of approximately four per minute. This regular contraction occurred in all preparations and is illustrated in Figure 3.2. The second most frequent, regular response was a periodic increase and decrease in amplitude of the contractions. This occurred in most preparations and is also shown in Figure 3.2. The frequency of these amplitude changes was generally between 3 and 5 minutes.

Statistical analysis of the amplitude and frequency of the contractions, and of changes in base pressure recordings before and after treatment, and subjective assessment of the pattern and type of contractions, demonstrated that neither proxicromil at 50 mg/kg nor atropine sulphate at 1 mg/kg (doses based on pre-death bodyweights) had affected the pressures developed within the stomachs.
Figure 3.2  An example of spontaneous stomach pressure changes in isolated rat stomachs

Contractions were initiated with 20 ml/kg (based on pre-death bodyweights) of 0.5% methyl cellulose. Two regular patterns of response are apparent; one with a frequency of approximately four contractions per minute and a second with a frequency of three to five minutes per contraction involving a regular increase and decrease in the amplitude of the contractions.
3.3.2 The effect of proxicromil on isolated fundic strip preparations

Responses of fundic strip preparations to histamine and proxicromil were recorded and analysed. Spontaneous contractions of the strips were obtained but these tended to fade over the duration of the experiments. Histamine at 400 µg/ml of organ bath fluid, (as the acid phosphate) induced contractions in two of the preparations and a relaxation in the third. The contractions were not inhibited by the presence of 2 µg/ml of mepyramine maleate. Proxicromil at 500 µg/ml and above, caused contractions in one preparation that had also contracted to histamine but had no effect in the second preparation that had contracted to histamine. A relaxation occurred after proxicromil in the preparation that had also relaxed in the presence of histamine. Proxicromil caused a relaxation after mepyramine maleate (at 2 µg/ml) in the preparation in which the drug had earlier had no effect. Neither histamine nor proxicromil abolished the spontaneous contractions of the tissues.

Thus it was demonstrated, in both this experiment and in that described in Section 3.3.1, that proxicromil had no direct inhibitory activity on the contractility of rat stomachs.
3.3.3 An in vitro preparation to demonstrate pyloric sphincter function

Figure 3.3 illustrates the rise in intra-luminal stomach pressure that occurred when a liquid was pumped slowly (2 ml/min) into the stomach. The pressure increased gradually until the pyloric sphincter opened and allowed the liquid to escape. Opening of the sphincter could also be seen visually by the escape of food debris from the open end of the duodenum. The rate of infusion had little effect upon the responses at rates between 1 and 3 ml/minute but at higher rates than this the pyloric sphincter remained open permanently and no pressure build-up could be achieved.

Table 3.1 shows the effect of infusions of proxicromil at 5 mg/ml compared to infusions with methyl cellulose. There were no marked differences between the peak pressure responses in the presence of proxicromil compared to those in the presence of methyl cellulose.
Figure 3.3 Intraluminal stomach pressures prior to and after opening of the pyloric sphincter in isolated rat stomachs.

The trace illustrates the maximum pressure developed within an isolated rat stomach before the pyloric sphincter opened. In this example proxicromil was infused at 1 ml/min and at 5 mg/kg (based on pre-death bodyweight).
Table 3.1  Peak pressures achieved whilst infusing proxicromil at 5 mg/kg compared to 0.5% methyl cellulose

Maximum pressures (mm of water) developed within isolated rat stomachs before the pyloric sphincters opened.

Mean pressures with standard errors shown.

Measurements made during infusions of either methyl cellulose or of proxicromil at a dose equivalent to an in vivo dose of 5 mg/kg, each at three different rates of infusion.

<table>
<thead>
<tr>
<th>Infusion Rate (ml/min)</th>
<th>Rat No</th>
<th>Peak Response (mm water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% Methyl Cellulose</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean 16.4 ± 3.2</td>
</tr>
<tr>
<td>Proxicromil 5 mg/kg</td>
<td>1</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean 17.5 ± 4.5</td>
</tr>
</tbody>
</table>
3.3.4 An in situ preparation to demonstrate functional parameters and the effect of proxicromil administered into the stomach or duodenum

When administered into the stomach, proxicromil had no effect on the spontaneous stomach contractions (See Figure 3.4). However, the drug caused an immediate and complete cessation of stomach contractions when it was administered into the duodenum (See Figure 3.5).

Table 3.2 shows group mean responses expressed as a change above or below the pre-test reading and illustrates the reproducibility of the responses to proxicromil administration.

3.3.5 Dose response relationship of proxicromil and the inhibition of gastric emptying in situ

Doses of proxicromil administered intraduodenally between 1 and 8 mg/kg induced inhibition of stomach contractions. However, the responses to proxicromil did not result in different degrees of inhibition. The drug, at whatever dose was used, caused either 100% inhibition or zero inhibition. The difference in responses with different doses was in the time taken to recovery.

Detailed results are shown in Table 3.3 and expressed graphically in Figure 3.6. A sample of a recording made from one rat is shown in Figure 3.7.
Figure 3.4 The effect of proxicromil on spontaneous stomach contractions after administration to a rat stomach in situ

The recording shows intraluminal stomach pressure changes. (Minutes marked at the top of the trace.)

Neither 0.5% aqueous methyl cellulose as control nor proxicromil (FPL 57787) at 50 mg/kg had any marked effect on the spontaneous contractions of the rat stomach.
Figure 3.5  The effect of proxicromil on spontaneous stomach contractions after administration to a rat duodenum in situ

The recording shows intraluminal stomach pressure changes before and after proxicromil (FPL57787) administration to the duodenum at 50 mg/kg. The immediate (less than 5 seconds) and complete cessation of spontaneous stomach pressure contractions occurred in all animals treated with this dose of proxicromil, (see Table 3.2). Recovery usually occurred within 30 minutes.
Table 3.2  The effect of proxicromil on spontaneous stomach contractions in situ

The results are shown as pressures (mm Hg) at the stated times after treatment, expressed as percentage changes from pressures recorded before treatment.

Each result is the mean of five consecutive contractions.

M/C = 0.5% methyl cellulose as vehicle and control

<table>
<thead>
<tr>
<th>Site of Treatment</th>
<th>M/C</th>
<th>Proxicromil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>Time after Treatment (min)</td>
<td>5</td>
</tr>
<tr>
<td>M/C</td>
<td>-19.5</td>
<td>20.1</td>
</tr>
<tr>
<td>Proxicromil</td>
<td>-23.6</td>
<td>39.3</td>
</tr>
<tr>
<td></td>
<td>4.7</td>
<td>73.5</td>
</tr>
<tr>
<td></td>
<td>-30.8</td>
<td>42.1</td>
</tr>
<tr>
<td>Duodenum</td>
<td>M/C</td>
<td>Proxicromil</td>
</tr>
<tr>
<td></td>
<td>Time after Treatment (min)</td>
<td>5</td>
</tr>
<tr>
<td>M/C</td>
<td>-3.5</td>
<td>-100.0</td>
</tr>
<tr>
<td>Proxicromil</td>
<td>9.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>-9.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>
Table 3.3  **Inhibition of stomach pressure changes to various doses of proxicromil administered into the duodenum**

Results are expressed as the duration of inhibition (min) following the stated dose of proxicromil. Means with standard errors (SE) indicated.

* The single dose of 16 mg/kg is not included in Figure 3.6.

N/A = Not administered

<table>
<thead>
<tr>
<th>Dose Rat (mg/kg) No.</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>35</td>
<td>70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>N/A</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>1.7 ± 0.2</td>
<td>3.6 ± 0.4</td>
<td>8.6 ± 0.8</td>
<td>19.4 ± 2.2</td>
<td>(70)</td>
</tr>
</tbody>
</table>
Figure 3.6  Dose-response relationship of proxicromil on inhibition of gastric emptying in rats

Mean responses with standard errors indicated.

The graph shows the linear relationship between the dose of proxicromil and the duration of inhibition of gastric emptying (as measured by inhibition of stomach contractions) after administration of proxicromil to the duodenum.

Data from Table 3.3
Figure 3.7  The effect of increasing doses of proxicromil administered to the duodenum on spontaneous stomach contractions in situ

The trace shows the inhibition of stomach contractions following the administration of proxicromil to the duodenum of an anaesthetised rat. The 'All or None' (the latter at 1 mg/kg) nature of the response to proxicromil is illustrated in this recording which also demonstrates the increasing duration of inhibition after increasing doses of proxicromil.
3.3.6 Investigation of the site of action of proxicromil in the gastro-intestinal tract

Some minor, partial inhibition of gastric contractions occurred after the administration of proxicromil wherever the intestinal cannula was inserted. However, total inhibition as seen in previous studies, was taken as the measurable response to the administration of the compound. The distances along the intestine at which this occurred are shown in Table 3.4. Complete inhibition of stomach contractions occurred when proxicromil was administered into the first 25 cm (approximately 25% of the intestinal length) of the small intestine. Inhibition of stomach contractions did not occur when the drug was administered beyond 30 cm from the stomach.

3.3.7 Effect of FPL 57787KA on stomach pressure changes in situ

The experiment was repeated in three rats. All animals showed cessation of stomach responses immediately after the administration of FPL 57787KA.

The soluble sodium salt, FPL 57787KA, showed the same activity on the stomach after administration to the duodenum as has been demonstrated previously with the insoluble acid, proxicromil (FPL 57787). Thus it can be concluded that the activity of proxicromil is not dependant on solid particulate compound resulting in a physical irritation in the duodenum.
Table 3.4  Duration of inhibition of stomach contractions after the administration of 8 mg/kg of proxicromil into the small intestine at various distances from the stomach

The table illustrates the lack of inhibitory effect of proxicromil on stomach contractions when the drug was administered into the duodenum more than 25 cm from the stomach.

* No inhibition of stomach contractions occurred.

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Cannula Insertion (cm from stomach)</th>
<th>Duration of inhibition of contractions (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>13</td>
<td>14</td>
<td>1.5</td>
</tr>
<tr>
<td>9</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>28</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>11</td>
<td>30</td>
<td>0*</td>
</tr>
<tr>
<td>1</td>
<td>43</td>
<td>0*</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>0*</td>
</tr>
<tr>
<td>3</td>
<td>80</td>
<td>0*</td>
</tr>
</tbody>
</table>
3.4 DISCUSSION

As proxicromil had such a marked inhibitory activity on gastric emptying in vivo it was anticipated that if an in vitro stomach preparation could be developed that showed spontaneous pressure changes then the drug would inhibit these contractions. A suitable in vitro preparation was developed that demonstrated very regular and reproducible contractions. The addition of proxicromil to this preparation however showed a notable lack of inhibitory activity. Thus it was concluded that the drug probably did not exert its activity by a direct effect on the stomach. As confirmation of this conclusion, the drug was also shown to have no inhibitory activity on the more conventional rat stomach strip in vitro preparation.

Because the drug did not directly inhibit stomach contractions the possibility that it caused a sustained contraction of the pyloric sphincter was investigated. Although the pyloric sphincter preparation would not make an ideal routine screening method due to the variability of response, it was clear that by considering all of the responses in the presence of methyl cellulose and proxicromil, and at all the infusion rates used, that there was no difference between these two treatments. The peak response in all the methyl cellulose-treated stomachs was 16.4 mm of water whilst that in the proxicromil treated stomachs was 17.5 mm of water (p >0.05).

Thus it was concluded that proxicromil did not exert its inhibitory activity on gastric emptying by a direct effect on the pyloric sphincter. This result, together with the previous finding that the drug did not have a direct effect on the stomach itself, suggested that the activity of the drug must be due to an indirect action that would not be demonstrated in vitro.
Studies to examine the effect of the drug on an *in situ* stomach preparation were therefore instigated. The administration of proxicromil to the stomach of each of these animals had no marked effect although there was a slight suggestion that it may have increased the contractility of the stomach. As this would have been in opposition to the known effect of the drug *in vivo* (ie. inhibition of gastric emptying) the possibility of this being a real effect was unlikely. Comparison of Figures 3.4 and 3.5 and the data in Table 3.2 shows very clearly the complete and immediate effect on the stomach of proxicromil administered into the duodenum. The drug completely inhibited the gastric contractions.

These results confirm previous suggestions, for example after X-ray photography (Chapter 2) that the drug does not inhibit gastric emptying whilst in the stomach, but does so only when present in the duodenum. There are a number of possible mechanisms by which this could occur. The drug could be absorbed from the duodenum and exert an effect directly on the stomach via the circulation. This possibility appears unlikely as the rapidity of the *in situ* inhibition was so marked.

Nevertheless the possibility has been examined and is described in Chapter 4. Alternatively the drug could have been triggering a reflex in the duodenum that fed back to the stomach to inhibit that organ. This is most likely the mechanism of action of the drug, but whether such a reflex is nervous or hormonal was not clarified in the studies described here. Chapter 4 examines these possibilities in more detail.
Part of the classical confirmation of a pharmacological action of a drug is the demonstration of a dose-response relationship. However with this drug and in this in situ preparation a conventional dose-magnitude of response relationship could not be established. The effect of proxicromil on the stomach pressure changes after administration to the duodenum appeared to be an 'all or none' response. However there was a dose-duration of response relationship that is shown graphically in Figure 3.6. This effect was probably related to the time taken for the drug to be removed from the sensitive portion of the duodenum, or to be so distributed that the concentration became ineffective.

To test this hypothesis, proxicromil was administered into the duodenum at various distances from the stomach. The rate of recovery after small doses of proxicromil had earlier indicated that the sensitive portion of the duodenum was not extensive. This was confirmed by the finding that the gastric inhibitory activity of proxicromil was restricted to the first 20 to 30 cm of the duodenum.

The possibility existed that the inhibitory activity of proxicromil was not due to a pharmacological activity but was a result of the physical presence of the insoluble acid. By inhibiting stomach contractions after the administration into the duodenum of the soluble sodium salt of proxicromil (i.e. FPL 57787KA) it was shown that the inhibition of gastric emptying was not due to particulate material.
In conclusion, this series of experiments has demonstrated that proxicromil exerts its inhibitory activity on gastric emptying by an unknown mechanism (but probably by a reflex) initiated in the first 20 to 30 cm of the rat duodenum, that the effect occurs immediately that the drug reaches the duodenum and that the duration of activity is related to the presence or concentration of the drug in the duodenum. Proxicromil had no direct effect on the gastric musculature nor on the pyloric sphincter.
CHAPTER 4

FURTHER STUDIES ON THE MECHANISM OF ACTION OF PROXICROMIL
4.1 INTRODUCTION

Previous studies described in Chapters 2 and 3 have shown that proxicromil caused inhibition of gastric emptying by an effect initiated in the duodenum. Although these results were consistent with a nervous reflex, it has not been conclusively shown that the drug does not exert its action by either the release of local hormones into the blood vasculature or as a result of absorption of the drug from the duodenum into the blood.

The first series of experiments described below examines the effect of intravenous administration of proxicromil. As other studies have shown that orally administered proxicromil in rats results in very low plasma concentrations but very marked inhibition of gastric emptying, intravenously administered drug should have a much greater effect on gastric emptying if absorption from the gastro-intestinal tract was necessary for activity. Also, to test the hypothesis that the drug exerted its action by releasing hormones into the blood vasculature, pairs of rats have been prepared with mixed blood circulations. To study the nervous reflexes involved, rats have been vagotomised, atropinised or had the central nervous system (CNS) partially or wholly destroyed.
4.2 METHODS

4.2.1 In situ inhibition of gastric emptying after intravenous administration of proxicromil

Previous studies have shown that proxicromil was only active in inhibiting gastric emptying when it was administered into the duodenum and that it had no activity when administered into the stomach and prevented from entering the duodenum. The possibility existed that the activity of the drug was due to it being absorbed into the blood from the duodenum and transported to the stomach where it could then exert its inhibitory activity directly. Although the time course of the response appeared to be too rapid (less than 5 seconds) for this to occur, the present study was designed to test the hypothesis that the presence of high concentrations of proxicromil in the blood would not cause a greater inhibition of gastric emptying than occurred after intra-duodenal administration.

The preparation of the animals was as described in detail in Chapter 3. Briefly, anaesthetised rats had cannulae inserted into the duodenum and the stomach and an in-dwelling catheter in the tail vein. Another cannula in the stomach was also included for measuring the pressure changes occurring there.

Contractions of the stomach were initiated with 20 ml/kg of 0.5% methyl cellulose (M450) administered into the stomach, and recorded on a Devices MX2 recorder. Proxicromil was administered intravenously via the in-dwelling tail catheter over periods of one to three minutes at a concentration of 12.5 mg/kg and at a dose volume of 1 ml/kg.
4.2.2 The effect of proxicromil on the stomach pressure changes in pairs of rats with a common blood circulation

Evidence has been presented earlier that the ability of proxicromil to inhibit stomach contractions after administration to the duodenum is probably due to an action on a nervous reflex. The possibility that the drug exerts its activity by the release of hormones or other chemicals into the blood vasculature, although unlikely, has not been completely eliminated. This study was therefore designed to show that if locally released chemical transmitters were responsible for this activity of proxicromil, a second rat with a common blood supply would be similarly affected.

Pairs of male rats (litter mates) (designated Rat A and Rat B) were anaesthetised with sodium pentobarbitone (Sagatal, May & Baker Ltd.) intravenously through an in-dwelling catheter in a lateral tail vein. This catheter was also used throughout the experiment for the administration of further quantities of sodium pentobarbitone in order to maintain adequate anaesthesia.

Both rats were opened along the abdominal mid-line and the oesophagus ligated immediately proximal to the stomach. A dual cannula (Cannulae X and Y, Figure 4.1) was implanted through the first centimetre of the duodenum and passed 1 to 2 cm into the stomach. In Rat A, an additional cannula (Cannula Z) was passed into the duodenum through the same opening as used to cannulate the stomach. In Rat B the duodenum was ligated immediately distal to the stomach cannula. The abdomens of both rats were then closed.
One end of an 18 cm long plastic cannula (PP50) filled with heparinized saline (500 units of heparin per ml of saline) was then inserted into the right jugular vein of Rat A and tied in place. The other end of the cannula was inserted into the right carotid artery of Rat B. A second cannula was inserted into the right carotid artery of Rat A and the right jugular vein of Rat B. Thus the cardiac output entering the right carotid artery of Rat A passed into Rat B and vice versa (See Figure 4.1). 0.1 ml/kg of heparinized saline was then administered via the in-dwelling venous catheters in the tails of the rats.

Methyl cellulose (0.5% aqueous) was then administered into the stomachs of both rats through Cannulae Y until regular pressure changes in the stomachs of both animals were recorded via Cannulae X through P23BB pressure transducers recording on an MX2 Devices recorder. Proxicromil at 20 ml/kg in 0.5% methyl cellulose, (0.1 mg/kg) was then administered into the duodenum of Rat A through the duodenal cannula (Cannula Z). Pressure recordings from the stomachs of both rats were continually recorded.

At the end of each experiment, 0.5 ml of a 10% Nigrosin-Eosin (50:50) aqueous solution was administered intravenously through the in-dwelling tail catheter of Rat A and the rapid darkening of the blood in the cannulae carrying blood between the two rats was taken as confirmation that blood was flowing freely between the two animals. Any experiments in which the dye did not appear in the connecting cannulae were discarded as in these cases clotting of the blood, in or around the cannulae, had occurred. The experiments were repeated until three pairs of rats were obtained that had not developed cannula-blocking blood clots during the course of the experiment.
Figure 4.1  Schematic representation of stomach, duodenum, venous and carotid cannulae in pairs of rats with a common blood circulation

Cannula X  Connected to a P23BB pressure transducer and MX2 Devices multi channel recorder.
Cannula Y  Stomach cannula for the administration of 0.5% methyl cellulose to induce contractions.
Cannula Z  Duodenal cannula for the administration of proxicromil.
4.2.3 The effect of atropine, vagotomy and destruction of the central nervous system on stomach contractions in rats and on the inhibitory effects of proxicromil

Proxicromil has been shown to inhibit stomach pressure changes in situ by a mechanism that probably involves nervous pathways. In order to determine more closely how this effect is achieved, a series of studies was undertaken in which the inhibitory activity of proxicromil was studied after either atropine sulphate, vagotomy or CNS destruction.

A. The effect of atropine sulphate on stomach pressure changes in situ

Male, Sprague Dawley rats were anaesthetised with intravenously administered sodium pentobarbitone and their stomachs prepared for in situ recording of pressure changes as described earlier. Atropine sulphate was administered either directly into the duodenum, into the stomach or intravenously. The activity of proxicromil was studied both before and after administration of the atropine sulphate. Atropine sulphate was administered at 0.5 mg/kg (0.5 mg/ml) to the duodenum, at 10 mg/kg to the stomach to give an equivalent concentration in that organ (ie. 0.5 mg/ml allowing for dilution by the 20 ml/kg of methyl cellulose present in the stomach to initiate contractions), or at 2 mg/kg intravenously. Proxicromil was administered at 8,16 or 20 mg/kg directly into the duodenum both before and after atropine sulphate administration. Treatment of individual animals is shown in Table 4.2.
B. The effect of vagotomy on stomach pressure changes and the effect on the responses to proxicromil

Anaesthetised rats were prepared for the recording of stomach contractions as described earlier. Anaesthesia was taken to a deeper level and the trachea cannulated. The animals were artificially respired with 1.8 ml of air per breath at a rate of 100 breaths per minute. Stomach pressure changes were recorded. The effect of bilateral vagotomy on the changes in pressure in the stomach were monitored. Proxicromil at 20 mg/kg was administered into the duodenum after the vagotomised rats had recovered from the immediate effects of vagotomy. Responses of the stomach before and after the compound were recorded.

C. The effect of partial or complete destruction of the central nervous system on stomach pressure changes and the effect on the responses to proxicromil

Anaesthetised rats were artificially respired with 1.8 ml of air per inspiration at a rate of 100 inspirations per minute. The rats were prepared for the measurement of pressure changes in the stomach and for the administration of drug directly into the duodenum. Two levels of CNS destruction were examined. One was complete destruction of the brain and spinal cord by pithing the animal. The second was complete destruction of the brain but not of the spinal cord. After destruction of the various parts of the CNS, the pressure in the stomach was monitored throughout each experiment. In the animals in which pressure changes in the stomach occurred after CNS destruction, proxicromil at 8 mg/kg was administered directly to the duodenum and the effect on stomach pressure changes recorded.
4.2.4 The effect of a local anaesthetic administered intraduodenally on the response of the stomach to proxicromil administered into the duodenum

Anaesthetised rats were prepared for the recording of stomach contractions with a cannula inserted into the proximal duodenum for the administration of drugs. The anaesthetised animals were left breathing spontaneously until regular stomach contractions were established. A large dose of xylocaine (10 mg/kg) was then administered into the duodenum. This procedure resulted in minor and inconsistent changes in the stomach contractions which however rapidly returned to a regular pattern.

Once a regular pattern of stomach contractions was established, proxicromil at 16 mg/kg was administered into the duodenum as a suspension in 0.5% methyl cellulose. Control animals were dosed intraduodenally with saline instead of the xylocaine and then received proxicromil at 16 mg/kg intraduodenally. Stomach pressure changes were recorded throughout the experiments.
4.3 RESULTS

4.3.1 In situ inhibition of gastric emptying after intravenous administration of proxicromil

Although the acute intravenous LD$_{50}$ for proxicromil in conscious rats is approximately 125 mg/kg, it was apparent from this study that one-tenth of this dose (i.e. 12.5 mg/kg) had adverse effects in anaesthetised rats, resulting in irregular respiration and a tendency to cause skeletal muscular spasms. These probably affected the stomach to some degree but, as described below, did not affect the outcome of the study.

The changes seen in frequency and amplitude after intravenous administration were considered to be related to the generalised acute toxicity of the compound following this route of administration. It was clear that intravenous administration of proxicromil was less effective in inhibiting gastric contractions than intraduodenal administration of the same dose. See Table 4.1.
Table 4.1  Results of proxicromil administration on pressure recordings in the stomach

A dose of proxicromil of 12.5 mg/kg was administered intraduodenally to Rat No. 3 as a positive control.

i.v. = Intravenous administration
i.d. = Intraduodenal administration

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Proxicromil (mg/kg)</th>
<th>Response of stomach pressure to proxicromil</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.5 i.v.</td>
<td>60% reduction in amplitude with increase in frequency. Partial recovery over one hour.</td>
</tr>
<tr>
<td>2</td>
<td>12.5 i.v.</td>
<td>Initial inhibition for 3 to 4 minutes with recovery and gradual reduction of contractions over a period of one hour.</td>
</tr>
<tr>
<td>3</td>
<td>12.5 i.v.</td>
<td>Some reduction in amplitude but increase in frequency.</td>
</tr>
<tr>
<td>3</td>
<td>12.5 i.d.</td>
<td>Immediate cessation of all stomach pressure changes.</td>
</tr>
</tbody>
</table>
4.3.2 The effect of proxicromil on the stomach pressure changes in pairs of rats with a common blood circulation

An example of the pressure recordings from one of the pairs of rats is shown in Figure 4.2. As can be seen, the stomach of Rat A immediately ceased contracting when proxicromil was administered to the duodenum. The stomach of Rat B continued to contract uninterrupted throughout the quiescent period of Rat A and during the recovery period of Rat A. These results demonstrated the absence of any circulating mediators released by proxicromil and thus confirmed the results from previous studies that the activity of the drug was due to a stimulation of a nervous reflex.
Figure 4.2  Typical stomach pressure changes in a pair of rats with a common blood circulation

Proxicromil was administered intraduodenally at 20 mg/kg to Rat A.

Stomach contractions of Rat A ceased immediately whilst those of Rat B continued.
4.3.3 The effect of atropine, vagotomy and destruction of the central nervous system on stomach contractions in rats and on the inhibitory effects of proxicromil

A. The effect of atropine sulphate and proxicromil on stomach pressure changes

The normal spontaneous rapid contractions of the stomach were inhibited by atropine sulphate administered intraduodenally or intravenously, See Table 4.2. Slow contractions were then initiated, (See Figure 4.3 a). Proxicromil administered intraduodenally inhibited these slow contractions.

B. The effect of vagotomy on stomach pressure changes and the effect of proxicromil on the responses

Vagotomy inhibited the normal spontaneous rapid contractions of the stomach and initiated slow contractions, See Table 4.3. However, proxicromil did not inhibit these slow contractions, See Figure 4.3 b).

C. The effect of partial or complete destruction of the central nervous system on stomach pressure changes and the effect of proxicromil on the responses

Destruction of the brain markedly reduced the activity of the stomach but did not inhibit rapid contractions completely nor did it result in the initiation of slow contractions, See Table 4.4. Proxicromil had little or no effect on the remaining contractions. Destruction of the brain and spinal cord inhibited all contractions of the stomach.
Table 4.2 Effect of proxicromil or atropine sulphate on spontaneous stomach contractions in situ

Proxicromil was administered intraduodenally to rats that had been treated with atropine sulphate intravenously, intraduodenally or directly into the stomach.

* i.s. = Administration into the stomach.  
  i.d. = Intraduodenal administration.  
  i.v. = Intravenous administration.

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Compound or drug</th>
<th>Route*</th>
<th>Dose (mg/kg)</th>
<th>Changes in normal Spontaneous Contractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Atropine</td>
<td>i.s.</td>
<td>10</td>
<td>Slow contractions induced</td>
</tr>
<tr>
<td></td>
<td>Atropine</td>
<td>i.d.</td>
<td>0.5</td>
<td>No additional effect</td>
</tr>
<tr>
<td></td>
<td>Atropine</td>
<td>i.s.</td>
<td>10</td>
<td>Normal contractions returning</td>
</tr>
<tr>
<td></td>
<td>Proxicromil</td>
<td>i.d.</td>
<td>8</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>Proxicromil</td>
<td>i.d.</td>
<td>16</td>
<td>No effect</td>
</tr>
<tr>
<td>2</td>
<td>Proxicromil</td>
<td>i.d.</td>
<td>8</td>
<td>Inhibition with recovery</td>
</tr>
<tr>
<td></td>
<td>Atropine</td>
<td>i.s.</td>
<td>10</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>Atropine</td>
<td>i.d.</td>
<td>8</td>
<td>Inhibition with recovery</td>
</tr>
<tr>
<td></td>
<td>Proxicromil</td>
<td>i.d.</td>
<td>0.5</td>
<td>Inhibition with recovery</td>
</tr>
<tr>
<td></td>
<td>Atropine</td>
<td>i.d.</td>
<td>8</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>Proxicromil</td>
<td>i.d.</td>
<td>0.5</td>
<td>Inhibition with recovery</td>
</tr>
<tr>
<td>3</td>
<td>Atropine</td>
<td>i.d.</td>
<td>0.5</td>
<td>Slow contractions induced</td>
</tr>
<tr>
<td></td>
<td>Proxicromil</td>
<td>i.d.</td>
<td>16</td>
<td>Inhibition</td>
</tr>
<tr>
<td></td>
<td>(See Figure 4.3 a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Atropine</td>
<td>i.d.</td>
<td>0.5</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>Atropine</td>
<td>i.d.</td>
<td>0.5</td>
<td>Slow contractions induced Partial inhibition</td>
</tr>
<tr>
<td></td>
<td>Proxicromil</td>
<td>i.d.</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Atropine</td>
<td>i.d.</td>
<td>0.5</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>Atropine</td>
<td>i.d.</td>
<td>0.5</td>
<td>Slow contractions induced Inhibition</td>
</tr>
<tr>
<td></td>
<td>Proxicromil</td>
<td>i.d.</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Atropine</td>
<td>i.d.</td>
<td>0.5</td>
<td>Little effect</td>
</tr>
<tr>
<td></td>
<td>Atropine</td>
<td>i.d.</td>
<td>0.5</td>
<td>Slower contractions induced Inhibition</td>
</tr>
<tr>
<td></td>
<td>Proxicromil</td>
<td>i.d.</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Atropine</td>
<td>i.v.</td>
<td>2</td>
<td>Inhibition with slow return</td>
</tr>
<tr>
<td></td>
<td>Atropine</td>
<td>i.v.</td>
<td>2</td>
<td>No additional effect</td>
</tr>
<tr>
<td></td>
<td>Proxicromil</td>
<td>i.v.</td>
<td>20</td>
<td>Inhibition</td>
</tr>
<tr>
<td>8</td>
<td>Atropine</td>
<td>i.v.</td>
<td>2</td>
<td>Inhibition then slow contractions</td>
</tr>
<tr>
<td></td>
<td>Proxicromil</td>
<td>i.d.</td>
<td>20</td>
<td>Slowly developing inhibition</td>
</tr>
<tr>
<td>9</td>
<td>Atropine</td>
<td>i.v.</td>
<td>2</td>
<td>Inhibition returning slowly</td>
</tr>
<tr>
<td></td>
<td>Proxicromil</td>
<td>i.d.</td>
<td>20</td>
<td>Brief inhibition</td>
</tr>
</tbody>
</table>
Table 4.3  Changes in spontaneous contractions after vagotomy and proxicromil

Proxicromil was administered at 20 mg/kg intraduodenally to rats that had been vagotomised.

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Effect of vagotomy on stomach pressure changes</th>
<th>Effect of intraduodenal Proxicromil at 20 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Contractions inhibited. Slow rise and fall in base-line initiated.</td>
<td>No effect</td>
</tr>
<tr>
<td>11</td>
<td>Contractions inhibited. Slow rise and fall in base-line initiated. See Figure 4.3 b)</td>
<td>No effect</td>
</tr>
<tr>
<td>12</td>
<td>All activity ceased.</td>
<td>Not administered</td>
</tr>
<tr>
<td>13</td>
<td>Contractions inhibited. Slow rise and fall in base-line initiated.</td>
<td>No effect</td>
</tr>
</tbody>
</table>
Figure 4.3a  Slow contractions induced by atropine sulphate and their inhibition by proxicromil

Atropine sulphate administered intraduodenally at 0.5 mg/kg.
Proxicromil administered intraduodenally at 16 mg/kg

Figure 4.3b  Slow contractions induced by vagotomy and the absence of inhibition by proxicromil

Slow contractions induced after bilateral vagotomy
Proxicromil administered intraduodenally at 20 mg/kg
Table 4.4  Responses of the stomach to CNS destruction and to proxicromil

Proxicromil was administered intraduodenally after the various sections of the central nervous system had been destroyed.

<table>
<thead>
<tr>
<th>Area of CNS destroyed</th>
<th>Rat No.</th>
<th>Response of stomach to CNS destruction</th>
<th>Response of stomach to Proxicromil at 8 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain only</td>
<td>15</td>
<td>Minimum activity</td>
<td>Not administered</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>Minimum activity</td>
<td>Not administered</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>Minimum activity especially in base-line</td>
<td>Not administered</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>Maximum-moderate activity</td>
<td>Minimal change</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Maximum-moderate activity</td>
<td>Not administered</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>Moderate-good activity</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>Good activity for three minutes then minimum</td>
<td>Not administered</td>
</tr>
<tr>
<td>Brain and spinal cord</td>
<td>14</td>
<td>No activity</td>
<td>Not administered</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>No activity but slight variation in base-line</td>
<td>Not administered</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>No activity</td>
<td>Not administered</td>
</tr>
</tbody>
</table>
4.3.4 The effect of a local anaesthetic administered intraduodenally on the response of the stomach to proxicromil administered into the duodenum

The administration of xylocaine to the duodenum induced minor and inconsistent responses in the stomach. These were primarily an initial decrease in contractility followed by irregular contractions. These irregularities tended to fade over a period of 15 to 30 minutes allowing regular contractions to become re-established. The irregular contractions were approximately 25% smaller in amplitude than those recorded prior to the administration of xylocaine.

The administration of proxicromil at 16 mg/kg; a dose shown previously, and confirmed in the control animals in this study, to induce 100% inhibition of stomach contractions; did not inhibit stomach contractions in the presence of xylocaine. There appeared to be a small degree of inhibition although this consisted of a re-introduction of irregularities in stomach contractions rather than an overall reduction in contractility.
The total (24 hour) absorption of proxicromil after oral administration to rats is approximately 40% (Fisons internal communication). Within five minutes of an oral dose the amount absorbed would be minimal (probably less than 0.1 μg/ml in the plasma). Even allowing for a possible faster absorption when the drug is administered directly to the duodenum rather than to the stomach, an intravenous dose of 12.5 mg/kg far exceeds the plasma concentrations likely to be encountered after 12.5 mg/kg intraduodenally. If proxicromil exerted its inhibitory action directly on the stomach after being absorbed into the blood, such an intravenous dose would therefore be expected to have at least as great an inhibitory activity as an intraduodenal dose. This was not the case and the results of these studies confirmed previous work (in vivo) that proxicromil administered intraduodenally does not require absorption to exert its inhibitory activity on gastric emptying.

The results of the paired-rats studies confirmed that proxicromil acts on the duodenum to inhibit stomach contractions by a mechanism consistant with a nervous rather than a hormonal action. If local hormones had been released by proxicromil and passed via the blood to the stomach, they would also have appeared in the circulation of the second rat and caused a similar cessation of stomach contractions, since to reach the stomach of Rat A from the duodenum of that rat the blood passes into the systemic circulation. There was no indication of any such transfer of hormones or other chemical entities between Rats A and B.
Further evidence of the nervous mechanism of the action of proxicromil in the duodenum was provided by the inhibition of the drug's activity by the prior administration of a local anaesthetic to the duodenum.

Thus it can be concluded, (considering also the evidence presented earlier), that proxicromil causes inhibition of stomach contractions and hence inhibition of gastric emptying in rats by a reflex initiated in the duodenum.

In a preceding chapter (Chapter 3), two spontaneous rhythms of the isolated stomach were described (see Figure 3.2 of Chapter 3). One of these rhythms was described as rapid contractions occurring approximately three times per minute. The second contraction, superimposed on the first, was a much slower rise and fall in the amplitude of contractions with a period of three minutes or more. This second, slower contraction has not been seen in situ in any of the numerous studies described earlier in which spontaneous contractions have always been of the rapid type and of which proxicromil has completely inhibited all activity (see Figure 3.5 of Chapter 3).

In the present studies however, both atropine sulphate (see Figure 4.3 a) and vagotomy (see Figure 4.3 b) abolished the rapid contractions and initiated much slower spontaneous responses of the stomach. Initially, vagotomy caused complete cessation of stomach contractions but gradually, over five to twenty minutes, contractions with a long periodicity developed. The slow contractions were not always of uniform pattern in different animals. Figures 4.3 a) and 4.3 b) show typical variations that occurred in both atropine-treated and vagotomised animals. The results of these studies suggest that
the rapid contractions are due to vagal stimulation and are abolished by either vagotomy or atropine sulphate, allowing the slower, locally induced contractions to occur. However, earlier studies have shown quite clearly that the rapid contractions also occur spontaneously in vitro without any vagal stimulation.

Proxicromil administered intraduodenally inhibited the slow contractions induced by atropine sulphate (see Figure 4.3 a). However in marked contrast to this, the drug did not inhibit the slow contractions induced by vagotomy (see Figure 4.3 b). This indicates that the inhibitory activity of proxicromil on these slow contractions depends upon a centrally mediated reflex that involves the vagus but does not involve the release of acetyl-choline at muscarinic receptors.

In conclusion, the inhibitory activity of proxicromil on the stomach has been shown to be due to a nervous reflex originating in the duodenum. The activity of the drug was clearly shown not to be due to circulating plasma concentrations of either the drug itself or of circulating hormones released by the drug. The reflex appeared to involve a non-cholinergic component of the vagus nerve.
CHAPTER 5

STRUCTURE-ACTIVITY RELATIONSHIPS

OF CHROMONES AND THE ACTIVITY OF

SOAPS AND BILE SALTS AS

INHIBITORS OF GASTRIC EMPTYING
5.1 INTRODUCTION

The design of drugs depends not only on their potential efficacy but also on their potential side-effects or toxicity. Within a related series of chemicals tested for their toxicity there usually arise a number of side-effects that appear with one or two chemicals but not with the majority. Other side-effects occur that tend to appear with most of the drugs and can, to a greater or lesser degree, be expected to occur. In the latter case it may be possible to establish some facets of structure-activity relationships that can be used to predict such side-effects before expense and time has been wasted on producing and testing a potential drug. Therefore an attempt was made to establish a structure-activity relationship between the ability to inhibit gastric emptying and the structures of a series of chromones.

Also reported in this chapter are experiments designed to determine a physiological role for the reflex that is triggered by proxicromil in the duodenum. For this purpose the effect of bile salts and soaps were studied for their ability to inhibit gastric emptying after administration to the duodenum.
5.2 METHODS

5.2.1 Structure-activity relationships of chromone compounds and inhibition of gastric emptying

The studies described here were designed to use the in vivo method of measuring gastric emptying described earlier to study a series of chromones and to attempt to establish a structure-activity relationship. The method for the estimation of gastric emptying rates by measuring stomach weights was as follows. All rats used in these experiments were male, Fisons-bred Sprague Dawleys that were surplus to Breeding Unit requirements. Up to three drugs were tested per experiment, each with five animals and each experiment included a group of five animals that were dosed with proxicromil as a standard and a group of 10 animals that were used as controls. Results were expressed as percentage activities compared to proxicromil in each study to enable comparisons to be made between experiments. All drugs were administered at 50 mg/kg in 0.5% aqueous methyl cellulose and dosed at 10 ml/kg in a random order between dose groups.

Six minutes after dosing the compound, 20 ml/kg of tap-water was administered orally. All rats were killed by an intraperitoneal injection of sodium pentobarbitone (Sagatal, 60 mg/ml) 90 minutes after the water was administered. The animals were opened along the abdominal mid-line and the oesophagus and duodenum clamped close to the stomach to prevent leakage of stomach contents. The stomach was then removed from the animals. The stomach and contents were immediately weighed and the weights expressed as percentages of total bodyweights.
The effect of soaps and bile salts administered into the duodenum on stomach contractions in situ

The animals were anaesthetised with sodium pentobarbitone (Sagatal, May & Baker Ltd.) administered intravenously after the animals had been warmed in a heated box. The rats were opened along the midline from immediately below the diaphragm for approximately 5 cm, thus exposing the abdominal contents. For intraduodenal administration, one short cannula was placed through a cut in the duodenum approximately 1 cm from the stomach and tied in place. A second cannula was used for recording the pressure in the stomach and was passed down the centre of a third cannula which was used for administration of methyl cellulose to the stomach. The end of this double cannula was placed approximately 1 cm inside the stomach and tied in place. All the cannulae were filled with 0.5% aqueous methyl cellulose before insertion and the exposed ends closed with three-way taps.

The oesophagus was then ligated immediately distal to the stomach to prevent regurgitation with a consequential loss in stomach pressure. The abdominal wall was then closed with Michel clips and the skin likewise. Intraluminal stomach pressure changes were recorded via a P23BB pressure transducer recording on a Devices MX2 recorder. To initiate stomach contractions, 0.5% aqueous methyl cellulose was administered to the stomach at 10 ml/kg. For administration of the soaps or bile salts to the duodenum a dose volume of 1.25 ml/kg was used. Lauryl sulphate and stearic acid palmityl ester were used as examples of esters of long chain fatty acids and taurocholate and deoxycholate as examples of bile salts. (Chemical formulae of these bile salts and soaps are shown in Table 5.6).
5.3 RESULTS

5.3.1 Structure-activity relationships of chromone compounds and inhibition of gastric emptying

The results, grouped into the separate experiments in which the particular drugs were studied, are presented in the Appendix (Table 2). Proxicromil as a positive control was used in each experiment. The results are summarized in Table 5.1 which also includes some results obtained with other drugs presented earlier in Chapter 2. The structures of all of the chromones studied are shown in Tables 5.2 to 5.5.

The activities of the chromones as inhibitors of gastric emptying varied greatly, from the most active FPL 52806 which resulted in virtual 100% inhibition over the 90 minute measuring period, to the completely inactive FPL 50670 (Intal). The implications of these results on the predictability of inhibition of gastric emptying for new chemical structures is considered further in the Discussion to this chapter.

Some chromones are known to have potent surface activity (detergency) properties. It was apparent during these experiments that such drugs, e.g. proxicromil itself, FPL 52758 and FPL 57579, were also very active inhibitors of gastric emptying. A similar association had also been apparent in other work carried out in the Toxicology Department, Fisons, between detergent properties and sensory irritancy to the respiratory tract. A statistical comparison of this sensory irritancy data with the inhibition of gastric emptying for the same chromones showed a highly significant correlation, \( p < 0.01 \). CS gas was included in that evaluation.
Table 5.1 Summary of the activity of chromone and other drugs as inhibitors of gastric emptying

* Not compared to proxicromil. Stomach weights less than control values and therefore only expressed as percentage change from control values.

** Non-chromone Fisons compounds.

<table>
<thead>
<tr>
<th>Compound/Drug</th>
<th>Activity as % of Proxicromil</th>
<th>p value v controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metoclopramide</td>
<td>-14.2*</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>FPL 59219KP</td>
<td>-14.0*</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>FPL 50670</td>
<td>-12.3*</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Sodium aspirin</td>
<td>-9.3*</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Aspirin</td>
<td>-0.5*</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>FPL 59002KP</td>
<td>3.1</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>5.7</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>FPL 55712LL</td>
<td>6.0</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>7.2</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>FPL 58935KP</td>
<td>15.7</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>17.1</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>FPL 59128AA</td>
<td>17.8</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>FPL 58302</td>
<td>21.5</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>FPL 56604KP</td>
<td>37.8</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>FPL 59112KA</td>
<td>38.3</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>FPL 57788AA</td>
<td>42.2</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>FPL 52694</td>
<td>44.5</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>FPL 52370</td>
<td>45.8</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>FPL 59038KA</td>
<td>49.1</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

(Cont...)
<table>
<thead>
<tr>
<th>Compound/Drug</th>
<th>Activity as % of Proxicromil</th>
<th>p value vs controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPL 58665KP</td>
<td>51.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FPL 58249KA</td>
<td>52.8</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>FPL 57978AA</td>
<td>57.6</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>FPL 58231AA</td>
<td>58.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FPL 52791</td>
<td>60.7</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>FPL 52757</td>
<td>60.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FPL 55687KA</td>
<td>71.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FPL 52693</td>
<td>74.6</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>FPL 57789KA</td>
<td>76.4</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>FPL 57950KA</td>
<td>79.0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FPL 57978AA</td>
<td>79.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FPL 55625KA</td>
<td>80.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FPL 55723KA</td>
<td>80.7</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>FPL 52758</td>
<td>85.0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FPL 58226</td>
<td>86.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FPL 58668KC</td>
<td>86.9</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>FPL 58852</td>
<td>92.2</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>FPL 55727KA</td>
<td>96.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FPL 57579</td>
<td>99.6</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Proxicromil</td>
<td>100.0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FPL 55618KA</td>
<td>101.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FPL 55731KA</td>
<td>103.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CS Gas</td>
<td>105.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FPL 52806</td>
<td>118.3</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
The following abbreviations are used in Tables 5.3 to 5.5 to describe the chemical structures of the chromones.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Chemical Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Et</td>
<td>Ethyl</td>
<td>-CH₂CH₃</td>
</tr>
<tr>
<td>Pr</td>
<td>Propyl</td>
<td>-CH₂CH₂CH₃</td>
</tr>
<tr>
<td>All</td>
<td>Allyl</td>
<td>-CH₂CH=CH₂</td>
</tr>
<tr>
<td>MeEt</td>
<td>2-Methylethyl</td>
<td>-CH(CH₃)₂</td>
</tr>
<tr>
<td>PropAc</td>
<td>Propionic acid</td>
<td>-CHCH₃COOH</td>
</tr>
<tr>
<td>t-Bu</td>
<td>t Butyl</td>
<td>-C(CH₃)₃</td>
</tr>
<tr>
<td>DiMeAm</td>
<td>Dimethylamino</td>
<td>-N(CH₃)₂</td>
</tr>
<tr>
<td>MeO</td>
<td>Methoxy</td>
<td>-OCH₃</td>
</tr>
<tr>
<td>PrPo</td>
<td>Propoxy</td>
<td>-OCH₂CH₂CH₃</td>
</tr>
<tr>
<td>HydPro</td>
<td>Hydroxypropoxy</td>
<td>-OCH₂CH(OH)CH₃</td>
</tr>
<tr>
<td>EtBuO</td>
<td>2-Ethylbutoxy</td>
<td>-OCH₂CH(CH₂CH₃)₂</td>
</tr>
<tr>
<td>MeBuo</td>
<td>2-Methylbutoxy</td>
<td>-OCH₂CH₂CH(CH₃)₂</td>
</tr>
</tbody>
</table>
| Tet          | Tetrazole     | \(\begin{array}{c}
\text{N}\hline
\text{N}\
\text{N}\end{array}\) |
| TetCarb      | Tetrazol-carboxamide (Amido-tetrazole)| -CO-NH\(\begin{array}{c}
\text{N}\hline
\text{N}\
\text{N}\end{array}\) + Na |
Table 5.3  Structure of chromones with the basic structure shown below

![Chemical Structure]

# FPL 57787 = Proxicromil
Sat = Saturated ring structure
Abbreviations used in the table below are defined in Table 5.2

<table>
<thead>
<tr>
<th>Compound FPL No.</th>
<th>2</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>10</th>
<th>Mol.Wt</th>
<th>Activity % Proxicromil</th>
</tr>
</thead>
<tbody>
<tr>
<td>57787# COOH</td>
<td>OH</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Pr</td>
<td>324</td>
<td>100</td>
</tr>
<tr>
<td>57579KA COONa</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Pr</td>
<td>308</td>
<td>100</td>
</tr>
<tr>
<td>58852 DiMeAm</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
<td>243</td>
<td>92</td>
</tr>
<tr>
<td>57978AA COOH</td>
<td>DiMeAm</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Pr</td>
<td>366</td>
<td>80</td>
</tr>
<tr>
<td>57950KA COONa</td>
<td>*</td>
<td>Sat</td>
<td>Sat</td>
<td>Sat</td>
<td>Pr</td>
<td>304</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>57789KA COONa</td>
<td>NH</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Pr</td>
<td>323</td>
<td>76</td>
</tr>
<tr>
<td>59038KA COONa</td>
<td>HydPro</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Pr</td>
<td>382</td>
<td>49</td>
</tr>
<tr>
<td>57788AA COOEt</td>
<td>NH</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Pr</td>
<td>348</td>
<td>42</td>
</tr>
<tr>
<td>59112KA COONa</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>MeBu0</td>
<td>352</td>
<td>38</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.4  Structure of chromones with the basic structure shown below

Notes 1 to 5 referred to in Table 5.4 are shown overleaf.

Abbreviations used in the table below are defined in Table 5.2

<table>
<thead>
<tr>
<th>Compound FPL No</th>
<th>2</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>MolWt</th>
<th>Activity % Proxicromil</th>
</tr>
</thead>
<tbody>
<tr>
<td>52806</td>
<td>COONa</td>
<td>*</td>
<td>t-Bu</td>
<td>*</td>
<td>t-Bu</td>
<td>324</td>
<td>118</td>
</tr>
<tr>
<td>55731 KA</td>
<td>COONa</td>
<td>*</td>
<td>Pr</td>
<td>*</td>
<td>Pr</td>
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<td>104</td>
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<td>55618 KA</td>
<td>COONa</td>
<td>MeBuO</td>
<td>*</td>
<td>*</td>
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<td>338</td>
<td>102</td>
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<td>97</td>
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<tr>
<td>58226</td>
<td>Pr</td>
<td>PrPo</td>
<td>NH</td>
<td>*</td>
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<td>Et</td>
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<td>COONa</td>
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<td>*</td>
<td>*</td>
<td>312</td>
<td>81</td>
</tr>
<tr>
<td>55625 KA</td>
<td>COONa</td>
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<td>*</td>
<td>*</td>
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<td>352</td>
<td>80</td>
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<tr>
<td>52693</td>
<td>COOH</td>
<td>HydPro</td>
<td>*</td>
<td>*</td>
<td>Pr</td>
<td>306</td>
<td>75</td>
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<td>55687 KA</td>
<td>Tet</td>
<td>MeBuO</td>
<td>*</td>
<td>*</td>
<td>All</td>
<td>362</td>
<td>72</td>
</tr>
<tr>
<td>52791</td>
<td>COOH</td>
<td>*</td>
<td>t-Bu</td>
<td>*</td>
<td>t-Bu</td>
<td>302</td>
<td>61</td>
</tr>
<tr>
<td>52757</td>
<td>COOH</td>
<td>OH</td>
<td>Et</td>
<td>*</td>
<td>Et</td>
<td>263</td>
<td>61</td>
</tr>
<tr>
<td>52694</td>
<td>COONa</td>
<td>HydPro</td>
<td>*</td>
<td>*</td>
<td>Pr</td>
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<td>45</td>
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<tr>
<td>56604 KP</td>
<td>TetCarb</td>
<td>MeO</td>
<td>*</td>
<td>See(2)</td>
<td>See(2)</td>
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<td>38</td>
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<tr>
<td>58302</td>
<td>Me</td>
<td>Et</td>
<td>*</td>
<td>ProPAc</td>
<td>*</td>
<td>*</td>
<td>262</td>
</tr>
<tr>
<td>55712 LL</td>
<td>See(3)</td>
<td>*</td>
<td>*</td>
<td>See(4)</td>
<td>Pr</td>
<td>644</td>
<td>6</td>
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<td>50670</td>
<td>COONa</td>
<td>See(5)</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>512</td>
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Notes to Table 5.4

1. 

2. 

3. 

4. 

5.
Table 5.5 Structure of chromones with the basic structure shown below

Notes to Table 5.5

1. 

2. 

3. 

4. 

Abbreviations used in the table below are defined in Table 5.2

<table>
<thead>
<tr>
<th>Compound FPL No.</th>
<th>2</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>Mol.Wt</th>
<th>Activity % Proxicromil</th>
</tr>
</thead>
<tbody>
<tr>
<td>58668KC</td>
<td>-</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>COOH</td>
<td>*</td>
<td>382</td>
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<td>58249KA</td>
<td>COONa</td>
<td>MeO</td>
<td>See Note 1</td>
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<td>53</td>
<td></td>
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<tr>
<td>58665KP</td>
<td>COONa</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>See(2)</td>
<td>*</td>
<td>387</td>
<td>51</td>
</tr>
<tr>
<td>52370KP</td>
<td>COONa</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>COONa</td>
<td>*</td>
<td>388</td>
<td>46</td>
</tr>
<tr>
<td>58935KP</td>
<td>TetCarb</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>TetCarb</td>
<td>*</td>
<td>522</td>
<td>16</td>
</tr>
<tr>
<td>59002KP</td>
<td>COONa</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>COONa</td>
<td>See(3)</td>
<td>415</td>
<td>3</td>
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<td>59219KP</td>
<td>COONa</td>
<td>*</td>
<td>Cl</td>
<td>*</td>
<td>COONa</td>
<td>See(4)</td>
<td>406</td>
<td>0</td>
</tr>
</tbody>
</table>
5.3.2 The effect of soaps and bile salts administered into the duodenum on stomach contractions in situ

As with proxicromil, the bile salts taurocholate and deoxycholate and the soaps lauryl sulphate and stearic acid palmityl ester produced 'all or none' responses on stomach contractions. The results (shown in Tables 5.7 and 5.8) are therefore expressed in terms of the duration of the inhibition, as was done for the dose-response curve established with proxicromil in Chapter 3.

Deoxycholate was generally more active in inhibiting gastric emptying than was taurocholate, although both induced complete inhibition of stomach contractions after administration to the duodenum. Potency was similar to that of proxicromil. (See Table 5.7)

The soaps lauryl sulphate and stearic acid palmityl ester were also inhibitors of gastric contractions after administration to the duodenum. The inhibition of gastric emptying induced by these detergents was rarely complete and was generally short-lived; the activity being less than that induced by proxicromil (See Table 5.8).
Table 5.6 Structural formulae of the bile salts, deoxycholate and taurocholate, and of the soaps, lauryl sulphate and stearic acid palmityl ester

Deoxycholic acid

\[ \text{C}_{23}\text{H}_{26}(\text{OH})_3\text{C}-\text{N}-\text{CH}_2-\text{CH}_2\cdot\text{SO}_3\cdot\text{H} \]

Taurocholic acid

\[ \text{CH}_3(\text{CH}_2)_{11}\cdot\text{SO}_3\cdot\text{Na}^+ \]

Lauryl sulphate (sodium dodecyl sulphate)

\[ \text{CH}_3(\text{CH}_2)_{16}-\text{C}-\text{CH}_2(\text{CH}_2)_{14}\cdot\text{COOH} \]

Stearic acid palmityl ester
Table 5.7  Inhibition of stomach contractions in situ following the intraduodenal administration of bile salts

<table>
<thead>
<tr>
<th>Bile Salt</th>
<th>Dose (mg/kg)</th>
<th>Duration of Inhibition (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
<td>None</td>
</tr>
<tr>
<td>Taurocholate</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>Deoxycholate</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>+20 partial</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>18</td>
</tr>
</tbody>
</table>
Table 5.8  Inhibition of stomach contractions in situ following the intraduodenal administration of soaps

<table>
<thead>
<tr>
<th>Soap</th>
<th>Dose (mg/kg)</th>
<th>Duration of Inhibition (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lauryl sulphate</td>
<td>20</td>
<td>&lt; 1</td>
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<tr>
<td></td>
<td>50</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt; 13 partial</td>
</tr>
<tr>
<td>Stearic acid palmityl ester</td>
<td>10</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>
5.4 Discussion

The chromone compounds studied in these in vivo experiments showed a range of activity as inhibitors of gastric emptying from the most active drug, FPL 52806 (activity v proxicromil = 118%, Table 5.4) to completely inactive drugs such as FPL 50670, (Intal, Table 5.4). Some of the chromone drugs such as FPL 59219KP may even be stimulators of gastric emptying (See Table 5.1). During this discussion such compounds are referred to as being inactive as inhibitors of gastric emptying.

With all the three basic chromone structures illustrated in Tables 5.3, 5.4, & 5.5, any substitution greater or less than the t-butyl or propyl groups caused a reduction in activity. When ethyl groups were introduced (e.g. in FPL 52757, activity = 61%) activity was reduced (c.f. FPL 52806 118% - t-butyl, and FPL 55731 104% - propyl) unless a larger group was also included, such as methylbutoxy (FPL 55727KA 97%) in which case activity was restored.

In two instances, the conversion of an acid to a sodium salt markedly increased activity (FPL 52791, 61% with its sodium salt, FPL 52806 118%, and FPL 52757 61% with its sodium salt FPL 52758 85%). However in a third acid/salt comparison the conversion of the acid to a sodium salt caused a decrease in activity (FPL 52693 75% with its sodium salt FPL 52694 45%). Also the conversion of a monocalcium salt (FPL 58668KC 87%) to a disodium salt (FPL 52370, 46%) dramatically reduced activity.

The removal of the hydroxyl group from the 5 position of proxicromil (100%) did not effect the activity (c.f. FPL 57579KA 100%) but the introduction of any group containing a nitrogen atom at the
5 position reduced activity, in some cases severely, (e.g. FPL 57978AA, dimethyl amino group, 80%; FPL 57789KA & FPL 57788AA NH group 76%, & 42% respectively. The introduction of a hydroxypropoxy group at this 5 position also dramatically reduced activity (FPL 59038KA 49%). The introduction of either a tetrazole or a tetrazole carboxamide moiety also reduced activity, (c.f. FPL 55618KA 102% with FPL 55687KA 72%; and FPL 58668KC 87% with FPL 58935KP 16%).

In summary, the structure-activity relationships have shown the following to reduce the possibility of chromone compounds exhibiting gastro-intestinal side effects. Substitutes at the carbon atom adjacent to the ring oxygen should be larger than propyl. The introduction of nitrogen at almost any position also appears to reduce the possibility of the drug being a potent inhibitor of gastric emptying. The use of tetrazoles and tetrazole carboxamide groupings can also be expected to reduce this side-effect. Whether the drug is an acid or a salt may influence its potential to inhibit gastric emptying but this influence can be in either direction and is thus not predictable.

Proxicromil has surface activity properties (Eason 1981) \(\text{EC}_{50}^* = 0.18 \text{mg/ml}\) that were considered as a possible mechanism for its (and other chromones) activity as an inhibitor of gastric emptying. *\(\text{EC}_{50}\) is the concentration required to lyse 50% of erythrocytes in a test mixture. The lower the \(\text{EC}_{50}\), the higher the surface activity.) For this reason, two soaps with known high surface activity properties were studied in situ. Lauryl sulphate for example has an \(\text{EC}_{50}\) as low as 0.04 mg/ml. Although both lauryl sulphate and stearic acid palmityl ester had activity as inhibitors of gastric emptying in situ when administered into the duodenum, their activity was not as marked as that of proxicromil and generally did not result
in a total 100% inhibition of stomach contractions. In addition, a correlation test between the in vivo activity of chromone compounds and their surface tension properties did not show a positive correlation, p > 0.05. Thus it was concluded that although a high surface activity appeared to contribute to an inhibition of gastric emptying, it was not this property alone that explained the activity of proxicromil.

The bile salts, taurocholate and deoxycholate were both active as inhibitors of gastric emptying in situ. Their activity was similar to that of proxicromil. The significance of this result and its relevance to the mode of action of proxicromil is discussed further in Chapter 6.

Other studies carried out in the Toxicology Department, Fisons plc, concerned with screening chromone drugs for their potential as sensory irritants in the lung, had shown that proxicromil had high sensory irritant properties. CS gas had been used as a positive control in these studies and was therefore included in the gastric emptying screen described in this chapter. As shown in Table 5.1, CS gas administered as a solid in suspension was very active as an inhibitor of gastric emptying. A correlation test between inhibition of gastric emptying induced by chromones and their sensory irritant properties showed a highly significant correlation (p < 0.01). The significance of this correlation is unclear but as both sensory irritancy and inhibition of gastric emptying are undesired side-effects for anti-allergic drugs, the avoidance of structures that are potential inhibitors of gastric emptying is also likely to lead to structures with lower potential as sensory irritants.
CHAPTER 6

DISCUSSION
Discussion

Zbinden, (1982) advocates the study of discrete organ systems or functions as a more scientific approach to toxicology than a general, non-specific screen as currently recommended by regulatory authorities. The work described in the preceding chapters fully supports such an approach and illustrates an example of the failure of conventional toxicity studies to demonstrate the potential of a drug to induce significant and certainly unacceptably frequent side-effects.

The compound studied in this thesis had been tested in almost one hundred toxicity studies (See Appendix, Table 1). These studies ranged from acute to life-time studies and covered every bodily function including the gastro-intestinal tract. The studies were almost all based on those required by regulatory authorities. However when the drug was administered to humans it induced side-effects that had not been predicted from this spectrum of conventional studies but which were of sufficient severity and incidence to consider withdrawal of the drug. This thesis demonstrates that with a very simple study these particular side-effects could have been predicted and illustrates how pharmaceutical toxicology could develop were it allowed to do so freely. The test developed during this work for example would have taken one person half a day, used ten animals and cost approximately one hundred pounds and would have been of more predictive value than the data generated from the six-month study that required two people for eight months, used over two hundred animals and cost approximately fifty-thousand pounds. This work does not indicate that long term chronic studies have no value (the
six-month toxicity study did generate other data relating to the safety of the drug) but it does illustrate that shorter term tests can have at least an equal value in predicting potential side-effects in humans.

A simple, quick (two to three hours) and reproducible method of demonstrating an inhibition of gastric emptying in vivo in rats has been developed. The method was shown to be suitable as a primary screen for detecting those drugs likely to induce gastro-intestinal side effects in humans. A second screen in marmosets, probably more predictive than the rat model, was also shown to be suitable to study those drugs high-lighted by the rat screen as being potential inhibitors of gastric emptying.

Proxicromil was shown to be a potent inhibitor of gastric emptying in both rats and marmosets - an effect that can be extrapolated to man as the likely cause of the gastro-intestinal side-effects reported in clinical trials. This effect was so marked in both animals and man that some explanation is required in order to understand how it could have failed to have been detected in the numerous toxicity studies carried out prior to clinical trials (See Appendix, Table 1). Some of the longer term chronic studies had included the measurement of food consumption and it could be expected that a severe inhibition of gastric emptying should in theory adversely influence both food consumption and consequently bodyweights. No such effect was seen. The explanation for this lack of effect on food consumption is probably what has been described as the "catching up phenomenon" such as occurs after exercise, during which gastro-intestinal motility is reduced but over 24 hours no overall effect is apparent. (Hellebrant & Miles, 1934).
Having failed to demonstrate any activity in vitro, and in order to study the inhibitory action of proxicromil on gastric emptying in more detail, an in situ preparation was developed that demonstrated the inhibitory effect of the drug on gastric emptying. This effect was shown quite clearly in both rats and marmosets to be induced only when the drug was present in the duodenum acting on a negative feed-back mechanism to control the rate at which the stomach contents, and hence further quantities of the drug, entered the duodenum.

It has been further demonstrated that proxicromil inhibits gastric emptying by stimulating a nervous reflex. This reflex almost certainly has a physiological role and has probably evolved for a particular purpose. Two possible physiological roles are considered most likely. One is as a defence against noxious substances that have been ingested. As discussed in Chapter 1, the stomach can be considered, along with its role as a reservoir, to be a major homeostatic organ. In evolutionary terms it could be envisaged that these two actions developed first, enzymic and acid activities developing later. Digestion would not have commenced until the food reached the intestine, into which there could be some control over the rate of entry.

The duodenum will generally only pass the food along the small intestine when it is in a suitable form, i.e. when the pH, osmolarity, glucose concentration, concentration of noxious substances etc. have all been reduced or changed to a level that is acceptable to the duodenum. To do this efficiently the duodenum must be able to control the rate at which the food leaves the stomach. As described in Chapter 1, mechanisms exist for controlling the rate at
which acids, fats, hypertonic solutions etc. enter the duodenum. There must almost certainly also be mechanisms for controlling the rate of entry of noxious substances, perhaps via irritant receptors, in order to reduce gastric emptying until duodenal secretions have sufficiently diluted, or otherwise neutralised, the contents to render them harmless.

It is possible that proxicromil stimulates a reflex, the physiological role of which is to control the rate of entry of noxious and irritant substances into the duodenum. This hypothesis is supported to a considerable extent by the positive correlation shown to exist between sensory irritancy in the lung and the ability to inhibit gastric emptying.

The second possible physiological role that proxicromil may be triggering is one that is normally stimulated by naturally occurring substances. One of the most important secretions of the duodenum that is required to achieve an acceptable milieu within the small intestine is the bile. If stomach contents enter the duodenum faster than the bile can neutralise or otherwise render the contents acceptable, then either the intestine could be damaged or food utilisation greatly reduced. Thus there must be a controlling mechanism to reduce the rate of gastric emptying. Is this control via the food or via the bile? Throughout the literature concerning the control of gastric emptying by the duodenum, the food contents are held directly responsible for this control. Cooke (1975) for example, states quite categorically:- "The rate of transfer of gastric contents to the small bowel is retarded by the activity of receptors sensitive to acid, fat etc...". Hunt & Knox (1968) have even demonstrated that fatty acids with differing chain lengths have
different affinities for fat receptors in the duodenum. In fact the presence of receptors in the small bowel responsive to acid, fat or any other food constituent has never been shown. It is probably more correct to say that, for example, fat in the duodenum results in a reduced rate of gastric emptying.

Contraction of the gall bladder in man begins approximately half an hour after eating (Davenport, 1977). This half-hour delay suggests that the stimulus occurs when the food, probably fat, enters the duodenum. It would be appropriate if a negative feed-back mechanism operated to prevent further gastric emptying occurring until the bile had completed its emulsifying and neutralising role on the chyme that had already entered the duodenum. Although such a mechanism is likely to exist in species with gall bladders, the absence of this organ in rats does not support this explanation.

Bile is produced and secreted continuously in rats but not necessarily with the same constituents in the presence and absence of fat. The secretion in rats of the two major components of bile, the bile acid-independent fraction and the bile acid-dependent fraction may be under separate control as they are in species with gall bladders. It is therefore possible that in rats the bile acid-dependent fraction is secreted preferentially in the presence of fat. This theory is supported by the work of Botham & Boyd (1983), who showed that the synthesis of bile acids was raised in rats fed on a fat-supplemented diet. Therefore the presence of fat in the duodenum probably stimulates the secretion of certain components of bile in all species. It is quite possible that the different chain lengths shown by Hunt & Knox (1968) to have different efficacies as inhibitors of gastric emptying actually have different efficacies as stimulators of bile flow. The stimulation of bile flow probably
occurs via the release of cytocystokinin as Maklouf (1979) has shown that this hormone causes gall bladder contraction in man and Geenen and colleagues (1980) have shown that CCK relaxes the sphincter of Oddi.

Thus a constituent of the bile could be responsible for the inhibition of gastric emptying and not the fat per se. Once again considering the evolutionary development of this reflex, there is a wide range of fats and fatty acids that any receptor would have to recognise. How much simpler it would have been to evolve receptors that only had to recognise a specific number of chemicals (or perhaps to recognise the surfactant properties of chemicals) that were present in the normal secretions of the duodenum when stimulated by the presence of fats?

In order to relate the action of bile to that of proxicromil the question was asked, "Are there any bile constituents likely to be inhibitors of gastric emptying that could be responsible for triggering such a reflex?". The most obvious candidates were considered to be the bile salts. In the studies reported in Chapter 5 two bile salts (taurocholate and deoxycholate) were therefore included. Both were shown to be very active inhibitors of gastric emptying in rats.

Thus it may be the bile salts and not fats per se that trigger the reflex which inhibits gastric emptying when fats enter the duodenum. Proxicromil and other active chromones could therefore exert their activity as inhibitors of gastric emptying by stimulating the same receptors that respond to the bile salts. Structurally there are many dissimilarities between the chromones and the bile salts and there is little justification for supporting the above hypothesis on the grounds of structural similarity.
However, although a positive correlation between the surface activity of the chromones and their activity as inhibitors of gastric emptying was not demonstrated, some of them, proxicromil in particular, have very strong surface activity properties - a property shared by the bile salts. The lack of a positive correlation with surface-activity may be due to different solubilities of the various compounds. In support of the hypothesis that proxicromil exerts its activity by stimulating the same receptors as the bile salts is that fat only results in an inhibition of gastric emptying whilst present in the upper intestine (Best, 1911), (c.f. acid - active in the upper and the middle intestine). Like fats, proxicromil was shown to be active only when it was present in the upper intestine.

Proxicromil may therefore stimulate a reflex that has a physiological role. However, it is not known whether this reflex is present as a defence against noxious substances or is a normal control of gastric emptying responsive to bile salts following stimulation of their release by fats. (See Figure 6.1)

Why does food not normally result in nausea and vomiting whereas proxicromil does if, directly or indirectly, they are acting on the same receptors? Food does result in nausea and vomiting when gastric emptying is delayed for several hours such as occurs during a gastro-intestinal infection. In vivo studies with proxicromil have shown that the drug also has a long duration of activity in inhibiting gastric emptying. This naturally results in the nausea and vomiting observed clinically. The cause of the long duration of activity of proxicromil is unknown but may be due to occupation of receptors for a long time or to an excessive stimulation of receptors resulting in a period of refraction.
Figure 6.1 Schematic representation of the possible mechanism of action of proxicromil-induced inhibition of gastric emptying.

Receptors. (+) stimulation. (-) inhibition.
A particularly interesting finding was the "all or none" effect of proxicromil on stomach contractions. These contractions equate with gastric emptying since McSwiney (1932) has demonstrated that fluid is expelled from the stomach in spurts which synchronise with the antral contractions. Assuming that proxicromil was excessively stimulating a physiological reflex, this indicates that normally the stomach is either contracting rhythmically to expel its contents into the duodenum or that these particular contractions are switched off by the reflex, rather than slowed down. There appears to be no reduced expulsion, i.e. the control of emptying is like a solenoid that is either opened or closed rather than the infinitely variable control obtained by a tap. Although the stomach is normally considered to have an infinitely variable ability to regulate the rate of emptying, the variability may be in the frequency of contractions rather than in their strength.

Because of the frequency of side-effects encountered with proxicromil in clinical trials, and human volunteer studies, an attempt was made (before the work reported in this thesis was carried out) to reduce the incidence by treating human volunteers with enteric coated tablets of the drug (Fisons, internal document). These tablets were designed so that the drug would not be released until it had left the stomach and entered the intestine. It was surprising, at that time, to find that the administration of enteric coated tablets did not reduce the frequency nor the severity of gastro-intestinal side-effects. As shown in the work reported in the preceding chapters, such a finding could have been predicted as the gastro-intestinal side-effects induced by proxicromil are induced only after the drug has left the stomach. These results in humans with enteric coated tablets are consistent with a duodenal action of proxicromil as has
been shown to occur in rats and marmosets and thus increases the confidence in the use of these animal screens to predict effects in man.

As discussed in Chapter 2, the rate of absorption of a drug can be markedly affected by its activity as an inhibitor of gastric emptying. This is not a new concept as for example Prescott (1974) described the reduced absorption of paracetamol following inhibition of gastric emptying, and Arnaud & Getaz, (1984) suggest that dose-dependant kinetics of orally administered caffeine may be due to a delayed gastric emptying and not to the previously believed limited capacity for caffeine metabolism. However the relevance of this fact to toxicity studies and the prediction of the results to man has received little attention.

FPL 52806 was shown in Chapters 2 & 5 to be an active inhibitor of gastric emptying in rats. During the toxicological evaluation of this drug rodents were used extensively in chronic studies of up to six months duration. As shown in Figure 2.12, plasma concentrations in rats as a result of the inhibition of gastric emptying showed a very slow rise to a low peak at four hours. As demonstrated in the work described in Chapter 2 in marmosets, and as determined by the lack of gastro-intestinal effects in humans, inhibition of gastric emptying does not occur in man and marmosets with this compound. Therefore the drug could enter the duodenum continuously from the stomach and thus be available for absorption which was shown in the marmoset to be rapid and to lead to high peak plasma concentrations.

Can the rodent toxicity studies carried out with FPL 52806 therefore be expected to be predictive for man? There are two ways in which plasma concentrations of a drug can influence the drug's potential to
cause toxicity. One is by a low, but continuous insult to an organ or cell; the second is by delivering a short-lived but severe insult. These are the two extremes. With FPL 52806 these two extremes probably occur in different species. In the rat a relatively low but sustained plasma concentration will give a low but continual insult whereas in marmosets and probably in humans, a higher peak concentration with a more rapid decline occurs.

Dose for dose, the rat could therefore be considered inappropriate as a species for predicting toxicity in man in chronic studies with this drug. However, because of the relatively non-toxic nature of the drug, a dose in rats up to 100 times the clinical dose could be administered without inducing overt toxicity. Thus compared to clinically achieved plasma concentrations, concentrations in rats many times greater occurred that were sustained for several hours. Thus adequate safety margins could be achieved. With drugs that cannot be administered to animals at doses greatly in excess of clinical doses because of a narrow therapeutic margin, an inhibition of gastric emptying in rats without such an inhibition occurring in humans would greatly reduce the predictive value of chronic toxicity studies carried out in this rodent species. With drugs such as proxicromil that inhibit gastric emptying in both rats and humans, the value and predictability of chronic rat studies will be much greater.

In addition, this work demonstrates that inhibition of gastric emptying, or the lack of it, can be a major cause of differing plasma profiles between species and may explain some cases of species variation in response to orally administered drugs. Different rates of rise of plasma concentrations after oral administration that are
often assumed to be due to different rates of absorption per se may in fact be due to different degrees of inhibition of gastric emptying. This may also explain some instances of variation of both toxicity and efficacy within a species, particularly in a heterogenous species such as man in which some individuals may be more susceptible to gastric inhibition than others. Hellebrandt & Miles (1934) for example have shown that in normal individuals the rate of transit through the gastro-intestinal tract can vary from 38 to 146 hours. Such a difference must influence the plasma profile of an orally administered drug and hence both its efficacy and potential toxicity.

The effect that inhibition of gastric emptying may have on efficacy has received minimal attention; its effect on toxicity has received even less. The effect on efficacy is likely to be similar to its effect on toxicity. Accepting homeopathy as an exception, both toxicity and efficacy depend on a drug being delivered to its site of action at a minimal concentration. The concentration of a drug at its site of action depends on the plasma concentration and thus for an orally administered drug on its rate of absorption. Studies reported in the preceding chapters have shown that the rate of gastric emptying is an important determinant of the rate of absorption.
Have the original objectives of this research programme been met?
In Chapter 1, the purpose of this work was stated as being to elucidate the mechanism by which proxicromil caused gastro-intestinal disorders in man, to determine whether the effect in humans could have been predicted from animal studies and, if so, to develop a simple but effective toxicity study that could be used with future drugs to predict their potential to induce this type of side-effect in humans.

To summarize, it is clear that these questions have been answered to varying degrees. Proxicromil causes gastro-intestinal disorders by a potent inhibitory activity on gastric-emptying. The drug exhibited this activity by stimulating a nervous reflex that probably has a physiological role either to protect the small intestine from noxious substances or to respond to naturally occurring substances such as the bile salts. It is firmly concluded from this work that this effect in humans could have been predicted. Simple, effective animal screens, a primary screen in rats and a more predictive secondary screen in marmosets, were developed that are now used within the Toxicology Department, Fisons, with new drugs to predict their potential to induce gastro-intestinal disorders in man.


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APPENDIX

PROXICROMIL TOXICITY STUDIES

THE IN VIVO ACTIVITY OF A SERIES OF CHROMONES

AS INHIBITORS OF GASTRIC EMPTYING

ANIMALS AND MATERIALS
Table 1  Proxicromil toxicity studies

All of the following studies were done as part of the safety evaluation assessment of proxicromil by the Toxicology Department, Fisons plc.

Those studies that were the responsibility of the author of this thesis are marked with an asterisk (*).

Except where indicated with a hatch (#) all the studies were carried out in the Toxicology Department, Fisons plc., Pharmaceutical Division during the period 1975 to 1981. Studies marked # were carried out externally under contract, * indicating that the author acted as Responsible Scientist on behalf of Fisons plc.

1. Acute oral observation study in rats and mice.
   * 2. A comparison of the acute oral toxicity of the acid and the salt of proxicromil in male mice.
   * 3. Acute oral toxicity study in rats and mice.
   4. The effect of aminophylline, ephedrine, isoprenaline, mepyramine, salbutamol, tolbutamide and sodium cromoglycate on the acute oral toxicity of proxicromil in mice.
   5. Acute intravenous observation study in mice.
   6. Acute intravenous toxicity in mice and rats.
   7. Acute oral observation study in male rats.
   8. Acute oral sitting study in rats.
   9. Acute observation study after intraperitoneal administration.
  10. Perivascular irritancy study in rats.

(cont....)
Table 1 (....cont)  Proxicromil toxicity studies

11. Acute oral toxicity in Dutch rabbits.
15. The effect of intravenous administration in conscious cats.
16. Effect on the gastro-intestinal motility of rats after oral administration.
17. Effect of an acute, or five consecutive daily oral doses, on sleep induced by pentobarbitone in mice.
18. Rotarod performance in mice after acute or multi-dose (4 days) oral administration.
19. The acute effect on locomotor activity in mice.
20. The effect on locomotor activity after ten days oral treatment in mice.
21. The effect on gastro-intestinal motility in mice after acute and multidose (4 days) oral administration.
22. The effect of an acute oral dose on the duration of ethanol induced sleep in mice.
23. The effect on body temperature of mice following oral administration.
24. The effect on gastro-intestinal motility in rats after acute oral administration.
25. Operant behaviour of rats after oral administration.
26. The effect on the cardiovascular and respiratory systems of anaesthetised cats.

(....cont....)
Table 1 (cont)  Proxicromil toxicity studies

27. Further studies of the effects on the cardiovascular and respiratory systems of anaesthetised cats.
28. The effect on the somatic reflexes of anaesthetised cats.
29. Effects on the cardiovascular and respiratory systems of anaesthetised squirrel monkeys after intravenous administration.
30. Further studies on the cardiovascular and respiratory systems of anaesthetised squirrel monkeys after intravenous administration.
31. Seven-day interaction study with ethanol in mice.
32. Twenty-one-day interaction study with hydrocortisone in rats.
33. Twenty-one-day interaction study with salbutamol in rats.
34. Twenty-eight-day interaction study with aspirin in rats.
35. Twenty-one-day interaction study with aminophylline and mepyramine in rats.
36. Twenty-one-day interaction study with ephedrine and salbutamol in rats.
37. Sixty-one-day oral study of general endocrine development in rats.
38. Four-day oral siting study in rats.
39. Twenty-eight-day oral study in rats.
* 40. Three-month oral study in rats.
*# 41. Six-month oral study in rats.
42. Preliminary oral study in dogs.
* 43. Twenty-eight-day oral toxicity study in dogs.
* 44. Twenty-eight-day supplementary oral study in dogs.
* 45. Three-month oral study in dogs.

(cont....)
Table 1 (...cont)  Proxicromil toxicity studies

* 46. Five-day oral study in squirrel monkeys.
* 47. Twenty-eight-day oral study in squirrel monkeys.
* 48. Three-month oral study in squirrel monkeys.
  49. Perivascular irritancy study in squirrel monkeys.
* 50. Twenty-eight-day oral study in Cynomolgus monkeys.
*# 51. Six-month oral study in Cynomolgus monkeys.
*# 52. Preliminary siting study in baboons.
*# 53. Six-month oral study in baboons.
  54. Genetic activity using the yeast Saccharomyces cerevisiae.
  55. Ames test.
  56. Assessment of mutagenic potential using \textit{in vitro} cytogenetic techniques.
  57. Micronucleus study in mice.
  58. Dominant lethal assay in mice.
  59. Assessment of mutagenic potential after multiple human dosing by analysis of metaphase preparations from lymphocyte cultures.
  60. Oral teratogenicity study in rats.
  61. Oral teratogenicity study in rabbits.
  62. Fertility and reproductive study in rats.
  63. Peri/post-natal study in rats.
  64. Sensory irritation study of the upper airways in mice after inhalation.
  65. Pulmonary irritation study in anaesthetised rabbits and cynomolgus monkeys after administration by aerosol.
  66. Pulmonary irritation study in anaesthetised rabbits after administration by aerosol.

(cont....)
Table 1 (....cont)  Proxicromil toxicity studies

* 67. A comparison of the toxicity of proxicromil with that of warfarin following multiple oral dosing to Wistar rats.
* 68. A comparison of the toxicity of proxicromil with that of warfarin following multiple oral dosing to Sprague Dawley rats.
* 69. Acute oral dosing compared to warfarin and sodium warfarin in rats.
* 70. Investigations of blood clotting times in rats dosed orally for twenty-eight days using two dietary levels of vitamin K.
* 71. Investigation of the haematuria caused by proxicromil after intravenous administration to rats.
* 72. Investigation of the effect of high doses on clotting times in rats.
* 73. Intravenous interaction study with vitamin K in rats.
  74. Evaluation of the effect on immunological function with particular reference to host defence mechanisms.
  75. Further studies of the influence of proxicromil on immunological function and host defence mechanisms.
  76. Determination of maximum tolerated dose in hamsters.
# 77. Carcinogenicity study in hamsters.
**# 78. Preliminary assessment of toxicity in rats after dietary administration for twenty-seven weeks.
**# 79. Carcinogenicity study in rats.
* 80. Preliminary multidose toxicity study in infant rats.
* 81. Thirty-five-day oral toxicity study in neo-natal rats.

(cont....)
Table 1 (....cont)  Proxicromil toxicity studies

The following studies were also done to assess the safety of the drug for it's potential use in the eye and on the skin.

* 82. Eye irritation of a 2% gel in rabbits.
* 83. Eye irritation studies in rabbits with a gel in rabbits.
* 84. Comparison of eye irritation of a gel and an aqueous formulation in rabbits.
* 85. One-day dermal study in rabbits.
* 86. Three-day dermal irritancy study in rabbits.
* 87. Twenty-eight-day dermal toxicity study in rabbits.
* 88. Twenty-eight-day dermal study in pigs.
* 89. Perivascular irritancy study in squirrel monkeys.
Table 2  The activity of a series of chromones as inhibitors of gastric emptying

The mean stomach weights as percentages of bodyweights are expressed as a percentage increase or decrease compared to control values. This result is then related to the activity of proxicromil to allow comparison between experiments.

Mean results with standard errors (SE) indicated.

$p =$ probability of the result being statistically different from the control value as assessed by the Student's 't' test.

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Stomach weights as % of B/wts (mean ± SE)</th>
<th>% change from controls</th>
<th>Activity as % of that of Proxicromil</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
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<td>+157 (100)</td>
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<tr>
<td>50</td>
<td>p &lt;0.001</td>
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<td>FPL 57579</td>
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<td>+156 100</td>
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<tr>
<td>50</td>
<td>p &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FPL 58852</td>
<td>4.21 ± 0.43</td>
<td>+145 93</td>
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<tr>
<td>50</td>
<td>p &lt;0.01</td>
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</tr>
<tr>
<td>FPL 58668KC</td>
<td>3.97 ± 0.39</td>
<td>+136 87</td>
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</tr>
<tr>
<td>50</td>
<td>p &lt;0.05</td>
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### Table 2 b) FPL 50670

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<th>Treatment (mg/kg)</th>
<th>Stomach weights as % of B/wts (mean ± SE)</th>
<th>% change from controls</th>
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<td>p &lt; 0.05</td>
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<td>FPL 50670 50</td>
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<td>No activity</td>
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<td>p &gt; 0.05</td>
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### Table 2 c) FPL 52806 and FPL 52791

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<th>Treatment (mg/kg)</th>
<th>Stomach weights as % of B/wts (mean ± SE)</th>
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<th>Activity as % of that of Proxicromil</th>
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<td>Proxicromil 50</td>
<td>4.35 ± 0.41</td>
<td>+111 (100)</td>
<td>(100)</td>
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<td>p &lt; 0.01</td>
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<tr>
<td>FPL 52806 50</td>
<td>4.77 ± 0.39</td>
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<td>p &lt; 0.001</td>
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<tr>
<td>FPL 52791 50</td>
<td>3.45 ± 0.47</td>
<td>+68 61</td>
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<td>p &lt; 0.05</td>
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Table 2 d) FPL 58231AA, FPL 58665KP and FPL 59128AA

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<th>Treatment (mg/kg)</th>
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<td>4.67 ± 0.65</td>
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<tr>
<td>FPL 58231AA 50</td>
<td>3.45 ± 0.31</td>
<td>+98 58</td>
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<td>p &lt; 0.001</td>
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<tr>
<td>FPL 58665KP 50</td>
<td>3.24 ± 0.20</td>
<td>+86 51</td>
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<td></td>
<td>p &lt; 0.001</td>
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<tr>
<td>FPL 59128AA 50</td>
<td>2.96 ± 0.28</td>
<td>+30 18</td>
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<td>p &gt; 0.05</td>
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Table 2 e) FPL 57950KA, FPL 57978AA and FPL 59002KP

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<td>Proxicromil 50</td>
<td>3.75 ± 0.16</td>
<td>+148 (100)</td>
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<td>p &lt; 0.001</td>
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<tr>
<td>FPL 57950KA 50</td>
<td>3.28 ± 0.11</td>
<td>+117 79</td>
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<td>p &lt; 0.001</td>
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<td>FPL 57978AA 50</td>
<td>2.80 ± 0.14</td>
<td>+85 58</td>
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<td>p &lt; 0.01</td>
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<tr>
<td>FPL 59002KP 50</td>
<td>1.58 ± 0.36</td>
<td>+5 No activity</td>
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<td></td>
<td>p &gt; 0.05</td>
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Table 2 f) FPL 52693, FPL 52694 and FPL 59219KP

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<th>Activity as % of that of Proxicromil</th>
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<tr>
<td>Proxicromil 50</td>
<td>3.66 ± 0.48</td>
<td>+112 (100)</td>
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<td>FPL 52693 50</td>
<td>3.17 ± 0.41</td>
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<td>FPL 52694 50</td>
<td>2.59 ± 0.19</td>
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<td>1.46 ± 0.19</td>
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Table 2 g) FPL 55712LL, FPL 52757 and FPL 58935KP

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<td>+160 (100)</td>
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<td>FPL 55712LL 50</td>
<td>1.71 ± 0.21</td>
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<td>FPL 52757 50</td>
<td>3.07 ± 0.28</td>
<td>+97 61</td>
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<tr>
<td>FPL 58935KP 50</td>
<td>1.95 ± 0.16</td>
<td>+25 16</td>
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Table 2) FPL 57788AA, FPL 58226 and FPL 59038KP

<table>
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<th>Activity as % of that of Proxicromil</th>
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<tr>
<td>FPL 58226 50</td>
<td>3.69 ± 0.55</td>
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<td>FPL 59038KP 50</td>
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Table 2) FPL 58249KA and FPL 55625KA

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### Table 2 j) FPL 52758LL

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<tr>
<td>Proxicromil 50</td>
<td>4.26 ± 0.25</td>
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<td>FPL 52758LL 50</td>
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### Table 2 k) FPL 58302

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<th>% change from controls</th>
<th>Activity as % of that of Proxicromil</th>
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<tr>
<td>Control</td>
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<td>Proxicromil 50</td>
<td>5.39 ± 0.55</td>
<td>+123 (100)</td>
<td></td>
</tr>
<tr>
<td>FPL 58302 50</td>
<td>3.06 ± 0.95</td>
<td>+26 22</td>
<td></td>
</tr>
</tbody>
</table>
Table 2 1) FPL 52370, FPL 56604KP and FPL 59112KA

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Stomach weights as % of B/wts (mean ± SE)</th>
<th>% change from controls</th>
<th>Activity as % of that of Proxicromil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.63 ± 0.15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proxicromil 50</td>
<td>4.27 ± 0.32</td>
<td>+162 (100)</td>
<td></td>
</tr>
<tr>
<td>FPL 52370 50</td>
<td>2.84 ± 0.45</td>
<td>+74.4 61</td>
<td>46</td>
</tr>
<tr>
<td>FPL 56604KP 50</td>
<td>2.63 ± 0.33</td>
<td>+61 38</td>
<td></td>
</tr>
<tr>
<td>FPL 59112KA 50</td>
<td>2.64 ± 0.23</td>
<td>+62 38</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 m) FPL 55618KA, FPL 55723KA and FPL 55727KA

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Stomach weights as % of B/wts (mean ± SE)</th>
<th>% change from controls</th>
<th>Activity as % of that of Proxicromil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.26 ± 0.12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proxicromil 50</td>
<td>4.62 ± 0.38</td>
<td>+104 (100)</td>
<td></td>
</tr>
<tr>
<td>FPL 55618KA 50</td>
<td>4.70 ± 0.25</td>
<td>+108 102</td>
<td></td>
</tr>
<tr>
<td>FPL 55723KA 50</td>
<td>3.73 ± 0.40</td>
<td>+65 81</td>
<td></td>
</tr>
<tr>
<td>FPL 55727KA 50</td>
<td>4.46 ± 0.27</td>
<td>+97 97</td>
<td></td>
</tr>
</tbody>
</table>
**Table 2 a) FPL 57789KA, FPL 57978AA and CS Gas**

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Stomach weights as % of B/wts (mean ± SE)</th>
<th>% change from controls</th>
<th>Activity as % of that of Proxicromil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.53 ± 0.23</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proxicromil 50</td>
<td>3.72 ± 0.49</td>
<td>+143 (p &lt; 0.001)</td>
<td>(100)</td>
</tr>
<tr>
<td>FPL 57789KA 50</td>
<td>3.21 ± 0.26</td>
<td>+110 (p &lt; 0.01)</td>
<td>76</td>
</tr>
<tr>
<td>FPL 57978AA 50</td>
<td>3.29 ± 0.34</td>
<td>+115 (p &lt; 0.001)</td>
<td>80</td>
</tr>
<tr>
<td>CS Gas 50</td>
<td>3.84 ± 0.30</td>
<td>+151 (p &lt; 0.001)</td>
<td>106</td>
</tr>
</tbody>
</table>

**Table 2 b) FPL 55731KA, FPL 55687KA and CS Gas**

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Stomach weights as % of B/wts (mean ± SE)</th>
<th>% change from controls</th>
<th>Activity as % of that of Proxicromil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.97 ± 0.04</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proxicromil 50</td>
<td>2.74 ± 0.18</td>
<td>+183 (p &lt; 0.001)</td>
<td>(100)</td>
</tr>
<tr>
<td>FPL 55731KA 50</td>
<td>2.84 ± 0.35</td>
<td>+193 (p &lt; 0.001)</td>
<td>104</td>
</tr>
<tr>
<td>FPL 55687KA 50</td>
<td>1.97 ± 0.22</td>
<td>+103 (p &lt; 0.001)</td>
<td>72</td>
</tr>
<tr>
<td>CS Gas 5</td>
<td>1.45 ± 0.13</td>
<td>+50 (p &lt; 0.001)</td>
<td>53</td>
</tr>
</tbody>
</table>
Table 3  Animals and materials

a) Animals

Rats  Sprague Dawley (S/D) male rats used throughout the experiments described in Chapters 2 to 5 were from the Toxicology Department, (Fisons plc.), Teratology Breeding Unit. All of the rats used were surplus to breeding requirements that would normally have been culled. All the animals were in good clinical condition.

Ferrets  The ferrets used were surplus to requirements for a twenty-eight-day toxicity study with a Fisons compound. These animals were supplied by a commercial supplier. The ferrets were clinically healthy before and during the study.

Marmosets  The marmosets used were also surplus to requirements for a toxicity study. The animals were supplied from the Fisons Marmoset Breeding Unit. The marmosets were clinically and behaviourally normal.
Table 3 (....cont) Animals and materials

b) Materials

Aspirin BP, Calmic Ltd.

Atropine Sulphate BP, Evans Medical Supplies Ltd.

Barium sulphate BP, Royal infirmary, Liverpool.

Cimetidine, (Tagamet), 100 mg/ml solution, Smith, Kline & French Laboratories Ltd.

Codeine Phosphate BP, Evans Medical Supplies Ltd.

Deoxycholic acid, Sigma Chemical Company.

Heparin sodium, 150 units/mg, Evans Medical Supplies Ltd.

Histamine acid phosphate, BDH Chemicals Ltd.

Ibuprofen, (Brufen), Boots Company.

Indomethacin, (Indocid), 5 mg/ml suspension, Thomas Morson Pharmaceuticals.

Lauryl sulphate, sodium salt, (Sodium dodecyl sulphate), Sigma Chemical Company.

Mepyramine maleate BP, May & Baker Ltd.

Methyl Cellulose, (Celacol), Grade M450, Batch No. 2960CT, Clinical Trials Dept., Fisons plc.

(cont....)
Metoclopramide monohydrochloride, (Maxolon), Beecham Research Laboratories.

o-Chlorobenzylidene-malonitrile, (CS Gas), Eastman Kodak Company.

Sodium pentobarbitone BP (Vet), (Sagatal), 60 mg/ml solution, May Baker Ltd.

Stearic acid palmityl ester, Sigma Chemical Company.

Taurocholic acid, Sodium salt, Sigma Chemical Company.

Xylocaine, (lignocaine hydrochloride), Astra Pharmaceuticals.

All Fisons (FPL) compounds were supplied by the Medical Chemistry or Clinical Trials Supplies Departments, Fisons plc. Proxicromil was supplied as a micronised powder.