CHARACTERIZATION OF RAT INTESTINAL IMMUNOREACTIVE MOTILIN (IR-M)

BY

Lee Vogel

B.Sc. University of British Columbia 1984

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

THE DEPARTMENT OF PHYSIOLOGY

UNIVERSITY OF BRITISH COLUMBIA

We accept this thesis as conforming to the required standards.

THE UNIVERSITY OF BRITISH COLUMBIA

APRIL, 1987

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Physiology

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date April 10th/87

SUMMARY

Interdigestive myoelectric activity in rat intestine has been recorded and characterized. The interdigestive pattern of activity can be disrupted by oral glucose and high doses of the duodenal ulcerogen cysteamine. Intravenous glucose had no effect on the interdigestive myoelectric pattern, nor did high doses of porcine motilin.

Attempts were made to develop a hybridoma cell line secreting antibodies that would recognize rat intestinal immunoreactive motilin (IR-M). The murine myeloma cell line NS1 was fused with murine B-cells primed against porcine motilin. One hundred of the monoclonal cell lines produced secreted monoclonal antibodies that recognized porcine motilin. Attempts to identify a cell line secreting antibodies with the ability to stain rat intestinal tissue, however, produced only negative results.

Rat intestinal IR-M has been characterized with respect to immunocytochemistry (ICC), radioimmunoassay (RIA), and chromatographic properties. The biological activity of partially purified rat intestinal IR-M has also been evaluated utilizing a rabbit isolated duodenal muscle strip preparation.

Five different antisera and one monoclonal antibody directed against natural porcine motilin were utilized in an effort to detect IR-M containing cells in rat intestinal tissues. A variety of techniques were employed including tissue fixation with either Bouins, paraformaldehyde, or benzoquinone. In addition a variety of staining methods including, fluorescein conjugated second antibody, peroxidase-antiperoxidase or peroxidase conjugated second antibody techniques

were used. All methods using these antibodies failed to detect IR-M in the rat small intestine.

Porcine motilin was able to displace ^{125}I-motilin from antisera 13-3, 72X and M03. These antisera were utilized in a motilin RIA to evaluate acid extracts of rat intestinal tissue for IR-M. Only antisera 13-3 and 72X were capable of detecting IR-M in gut extracts, and these antisera gave different distributions of IR-M in the proximal small bowel.

Rat intestinal tissue was extracted into 2% trifluoroacetic acid and the soluble fraction clarified by centrifugation. This acid extracted material was precipitated with sodium chloride then dissolved in methanol at pH 6.0. Methanol soluble material was precipitated with ether and the ether precipitate then dissolved in water and chromatographed on Sep-Pak C_{18} cartridges (Waters). Sep-Pak cartridges were eluted with 50% acetonitrile: 0.1% TFA. The 50% eluate was then fractionated further using cation exchange, gel filtration and reverse phase high pressure liquid chromatography (HPLC).

Rat intestinal IR-M peaks from cation exchange chromatography on SP-Sephadex-C25 (Pharmacia) were concentrated and examined for contractility in a rabbit duodenal muscle strip preparation. Purification after SP-Sephadex-C25 was approximately 20 fold. Desensitization of rabbit duodenum to porcine motilin could be demonstrated by pre-treatment with motilin. Contractile activity of partially purified rat intestinal IR-M was not inhibited by pre-treatment with motilin.

Chromatography on Bio-Gel P-10 (Biorad) eluted with 0.2M acetic acid yielded an IR-M peak co-eluting with natural porcine motilin. On

HPLC, using a linear gradient of water/acetonitrile (10-45% acetonitrile in 30 min), rat intestinal IR-M did not co-elute with natural porcine motilin.

In conclusion, the molecular weight of rat intestinal IR-M appeared to be similar to porcine motilin as these two substances demonstrated co-elution on gel permeation chromatography. The lack of co-elution with porcine motilin on HPLC indicates that other molecular characteristics of rat intestinal IR-M, such as hydrophobicity, are not similar to porcine motilin. Furthermore, partially purified rat intestinal IR-M did induce a contractile response in rabbit duodenal muscle strips but porcine motilin did not desensitize this preparation to the contractile activity of rat intestinal extracts. This suggests that the contractile activity of these two compounds is induced via different receptor mechanisms. It is concluded that the immunoreactive motilin found in rat intestinal extracts does not resemble natural porcine motilin in structure or biological activity.

CONTENTS PAGE

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The extrinsic and intrinsic neural circuitry involved in intestinal motility is now relatively well established. The three types of autonomic nerves involved in the control of gastrointestinal motility are the parasympathetic nerves, the noradrenergic nerves and the non-adrenergic non-cholinergic (NANC) inhibitory and excitatory nerves (100). In general acetylcholine acts as an excitatory neuromuscular transmitter within the GI tract, whereas postganglionic sympathetic activity primarily suppresses motility by inhibiting acetylcholine release (100).

Descending inhibition was first described by Bayliss and Starling in 1899 (2,3). It is now generally accepted that in the stomach and small intestine a wave of relaxation propagates aborally ahead of the zone of contraction. Receptive relaxation of the stomach facilitates the entry of food from the oesophagus. In the intestine descending inhibition is an integral part of the peristaltic reflex pathway that facilitates the aboral movement of chyme. It has been evident for several years that the neural pathways involved in descending inhibition are NANC in nature. Recently, considerable attention has been given to vasoactive intestinal peptide (VIP) containing neurons and their role in the enteric nervous system as possible mediators of descending inhibition. VIP containing neurons in the guinea pig have been shown to project in an aboral direction at virtually all levels of the GI tract (35,36). Furthermore, VIP and its co-synthesized

analogue PHI appear to be the only neural peptides that cause direct relaxation of smooth muscle, and are contained in neurons projecting from the myenteric plexus to the smooth muscle layers (39,57). These observations are part of a growing body of evidence suggesting that VIP is the major inhibitory transmitter in reflex inhibitory pathways of the gastrointestinal tract. Other enteric neuropeptides such as neurotensin, and somatostatin, when administered exogenously in small doses, also produce an inhibitory effect on gastrointestinal smooth muscle activity (100). Myenteric somatostatin neurons, however, do not project beyond this plexus (52). Thus, it appears as though somatostatin may have an indirect role in regulating smooth muscle function possibly by inhibiting acetylcholine release from myenteric cholinergic neurons as well as by modulating the release of inhibitory peptides (57).

Studies utilizing isolated cross-perfused stomach preparations have provided evidence that motility patterns are also dependent on hormonal mechanisms (73). When the isolated stomach was perfused with blood from a fed animal the gastric motility pattern resembled that seen in the <u>in vivo</u> fed state. Conversely, the fed pattern of motility was abolished when the isolated stomach was perfused with blood from a fasted animal. Several known gastrointestinal hormones may play a role in the humoral mediation of motility patterns. Two such hormones, gastrin and cholecystokinin (CCK), are known to be released following ingestion of a meal and can delay gastric emptying as well as disrupt the fasting pattern of motor activity in the proximal small bowel. The only intestinal peptide that is both

inhibited by ingestion of a meal and shows enhanced release during the fasted state is motilin. The physiological role of this peptide remains controversial, but it appears to be associated with the regulation of interdigestive gastrointestinal motility.

The original isolation of motilin resulted from attempts to identify the stimulus responsible for increased gastric motility seen with alkalinization of the duodenum (14). Early experiments in man had demonstrated that duodenal perfusion with a 1% sodium bicarbonate solution induced rapid gastric emptying (86,87). During the same period Thomas et. al. (93) demonstrated an increase in gastric motor activity and a concomitant decrease in gastric emptying time when gastric contents were diverted from the duodenum. In dog experiments instillation of alkaline buffer or porcine pancreatic juice into the duodenum was found to elicit motor activity in the extrinsically denervated stomach (12). These results clearly indicated that a humoral mechanism was involved but the distinction between release of a stimulatory agent or inhibition of an inhibitor was not apparent. Several commercially available duodenal extracts were assayed for gastric motor-stimulating activity (9). A pancreozymin preparation (Boots Pure Drug Co. U.K.) demonstrated a powerful motor stimulatory activity. It was subsequently shown that the motor stimulating ability could be separated from the pancreozymin activity (14), thus demonstrating that this crude material contained a separate motor stimulating factor. The isolation and characterization of the motor stimulating factor from this Pancreozymin preparation proved unfeasible. However, side fractions produced during the isolation of

secretin from hog duodenal mucosa (62) were extracted and the purified extract sequenced (10). This revealed a 22 amino acid polypeptide that was named motilin on the basis of its stimulatory activity in fundic pouches (10,11,13). Although it was not known at the time the term motilin had been introduced previously (28,79) for a hypothetical agent with the ability to induce intestinal peristalsis (64).

Once the complete amino acid sequence of motilin was known* (11), it was synthesized (103) and both the synthetic and natural forms have been used to study the actions of motilin and to develop antibodies for radioimmunoassay (RIA) and immunocytochemical (ICC) studies. Motilin-like immunoreactivity (IR-M) has been demonstrated in a variety of mammalian species. However, the only other species in which this peptide has been isolated and sequenced is the dog. Canine motilin differs from porcine motilin in 5 of its 22 amino acids (75,80). This represents a relatively large species variation in the amino acid composition of motilin as other intestinal peptides such as VIP, somatostatin and secretin show no variation between mammalian species characterized to date. The fact that porcine motilin has been utilized to generate antisera to investigate the physiology of motilin in other animal models may underly much of the controversy relating to the origin, action, and release of this peptide.

Immunocytochemical studies have demonstrated motilin positive cells in the upper small intestine of several species, but the specific cell type has been the subject of debate. One group, using antisera raised against natural porcine motilin, has described

*The question of whether the identification of glutamic acid at position 14 is an artefact or represents heterogeneity of the natural peptide remains unresolved (88 b).

motilin positive cells which represent a subpopulation of enterochromaffin (EC) cells distinct from substance P containing EC cells (43,71,78). More recently, subcellular fractionation of human jejunal cell homogenates has confirmed the existence of two subtypes of EC cells (26): EC-1 cells containing substance P, and EC-2 cells containing motilin. Another set of studies utilizing antisera raised against synthetic porcine motilin failed to detect motilin in EC cells of any type (30,44). These latter observations led to the revision of the Lausanne classification of gastro-enteropancreatic endocrine cells to include the designation Mo for specific motilin cells (89,90).

Controversy also surrounds the reported localization of motilin in CNS tissues. Radioimmunoassay and immunocytochemical data have described relatively large concentrations of motilin in cerebellar tissue (19,20,21,66). A more recent report, employing several antisera, failed to find any IR-M peaks when screening chromatographed extracts of rat cerebellum (54). In addition, the same work indicated that only one of the eight antisera used showed ICC staining of the cerebellum. This staining, however, was not blocked by pre-incubation with motilin. These results suggest that ICC localization of motilin in the cerebellum may result from non-specific binding of an uncharacterized fraction of the antisera. In support of this view it has been observed that several monoclonal antibodies raised against porcine motilin do not stain CNS tissue in a variety of species including the rat (S. Vincent personal communication).

The stimulus for the release of motilin and its physiological role in the gastrointestinal tract are two areas that are also poorly understood as the two primary models for study of this peptide (man and dog) have yielded only equivocal data. Oral or intravenous fat stimulated motilin release in man (23), but had no effect on motilin release in dogs (61). Mixtures of amino acids inhibit motilin release in both man and dog (23,61). However, a mixed meal produces either minor increases or no change in motilin levels in man and pig (22,60). Similarly, motilin infusion in dogs speeds gastric emptying of liquid but not solid meals, and has the opposite effect in man, where gastric emptying of solids but not liquids is enhanced (23,27,82).

Motilin appears to act in the gut by modulating the release of acetylcholine as well as an unknown non-adrenergic non-cholinergic excitatory transmitter. The actions of motilin in the gut have been most thoroughly studied *in vivo* in association with the migrating motor complex (MMC) (see below). This naturally occurring complex appears to be induced by motilin and is blocked by atropine and tetrodotoxin (68,83), suggesting that motilin acts to release acetylcholine and/or other neurotransmitters. This action may also vary at different levels of the gastrointestinal tract. Motilin induced activity in the canine stomach *in vivo* (31,32) or *ex vivo* (25) was reduced by hexamethonium and totally blocked by tetrodotoxin or atropine. In the small intestine atropine alone produces only a partial blockade of motilin induced activity. Tetrodotoxin or a combination of atropine and hexamethonium are required to abolish the

response (31,32). These results suggest that the action of motilin in the stomach is mediated entirely by a cholinergic mechanism, whereas in the small intestine it is via both cholinergic and non-cholinergic pathways. It has been suggested that the non-cholinergic excitatory transmitter acting in the small intestine is opioid in character and possibly met-enkephalin (30).

A more recent report utilizing the isolated perfused canine small intestine has demonstrated that motilin-induced contractile activity is effectively antagonized by both tetrodotoxin and atropine (45). Furthermore, perfusion of the bowel segment with naloxone did not affect the response to motilin. The conflicting results reported for either *in vivo*, *in situ*, or *ex vivo* preparations appear to reflect the degree to which external neural elements are left intact.

There are some data indicating that motilin has a direct action on intestinal smooth muscle. These data have come from *in vitro* experiments, and are almost exclusively derived from studies with isolated strips of rabbit intestinal muscle. Tissues from all other species except man are refractory to motilin (1,85,91). In the rabbit, contractile responses of muscle strips *in vitro* were not affected by neural blocking agents or antagonists to several transmitter substances. Calcium antagonists or incubation in a calcium free medium abolished the response to motilin (1). These results suggest that in the rabbit motilin acts directly on smooth muscle, presumably by enhancing calcium influx. A recent study has reported the specific binding of motilin to homogenates of rabbit intestinal tissue (15). Subcellular fractionation indicated that

binding occurs at the plasma membrane. The relative distribution of motilin receptors to neural elements or other tissue types within the homogenate was not examined. It was suggested, however, that because of the very high amount of binding some of the receptors must be localized to the smooth muscle cells (15).

The effect of motilin on rabbit tissue in vitro may reflect a species difference. However, the isolated rabbit duodenal muscle strip exhibits a high degree of basal contractility in comparison to other species. Consequently it has been suggested that increased neuronal activity associated with a high level of basal contraction, as seen in the rabbit, may be required to observe an effect with motilin in other species (58).

The focus of attention concerning the physiology of motilin has been its association with phasic contractile activity during the fasted state. The first detailed investigation of myoelectric activity associated with interdigestive phasic contractions was by Szurszewski in 1969 (92). This pattern of activity originates in the stomach or upper small bowel and propagates distally to the ileum, thus the name migrating myoelectric complex. These myoelectric events associated with the interdigestive period in man have since been characterized into four recurring phases. Phase I corresponds to a period of quiescence lasting 60-70 minutes. Phase II is a period of intermittent contractions of 15-25 minutes. Phase III is a 4 to 6 minute period of regular strong contractions, followed by phase IV, a brief transition phase back to quiescence, the overall period of the MMC cycle being 90-100 minutes (100). The variations in the

myoelectric activity occurring from phase II though phase IV have also been termed activity fronts.

In man and in dog several groups found cyclic variations in plasma motilin levels that peaked in association with phase III of the MMC (49,50,95,96). This correlation between motilin peaks and interdigestive myoelectric activity has stimulated considerable research into a possible role for motilin in the regulation of MMC's. The primary research model in this case is the dog, in which the following relationships have been observed. Peaks of IR-M are associated with passage of an activity front through the duodenum (76). This association is independent of vagal activity (42). Activity fronts initiated in the jejunum show no correlation with peaks in plasma IR-M (70). Intravenous infusion of motilin in fasted dogs will initiate contractile activity resembling premature activity fronts (48,101). Phasic activity remains associated with endogenous IR-M peaks in denervated fundic pouches (94), but not in autotransplanted jejunal loops (84). Finally, it has been demonstrated that low doses of motilin antisera will temporarily inhibit the MMC in the antrum and small bowel, while larger doses induce prolonged inhibition (55,74). The conclusion that must be drawn from all these data is that motilin does play an active role in generating or modulating activity fronts in the stomach, at least in the dog. Motilin, however, does not appear to be involved in the propagation of activity fronts aborally from the duodenum to the terminal ileum. Evidence for a similar action of motilin in other species is less conclusive except, surprisingly, in the pig where

motilin does not appear to have any association with interdigestive phasic activity (17).

An important question that has received little attention to date is: what factors influence the cyclic pattern of motilin release? It is apparent that the major site for release of motilin in the dog is the upper small intestine and the only motilin dependent segment of the MMC is the gastric component. Consequently, it is most likely that the motilin released from the upper small intestine is having an endocrine effect at the level of the stomach. One group has suggested that motilin release is secondary to smooth muscle contraction (30). However, this does not explain how plasma IR-M peaks coincide with gastric phase III activity when the stomach has been shown to contain few if any motilin cells (6,71). Plasma IR-M concentrations were increased by bilateral vagal blockade in conscious animals (42), and decreased by vagal stimulation (sham feeding) (56). These observations suggest that cyclic patterns of release may be regulated primarily by vagal inhibitory pathways.

As outlined above, virtually every aspect of motilin physiology remains controversial. Most of the differences probably result from the use of a number of different antisera which undoubtedly display widely varying specificities for porcine motilin. Interspecies differences (5 amino acid difference between porcine and canine motilin) may account for variability in detectable levels of immunoreactive motilin (IR-M). Similarly, infusion of porcine motilin in non-porcine models may produce effects different from those caused by the endogenous hormone. Furthermore, the most prominent feature

associated with motilin release, the MMC, is only observed *in vivo*. Presently, the only adequately characterized *in vivo* model for motilin is in the dog. It is apparent that a well characterized rodent model is needed to help solve the enigma that this peptide has presented. The interdigestive migrating myoelectric complex has been observed in the rat (72,81,99). In addition it has been observed that intraduodenal administration of glucose and amino acids disrupt the MMC in rats (81). Subcutaneous administration of morphine sulfate (99) and intravenous administration of duodenal ulcerogens, cysteamine and proprionitrile (72) have also been shown to disrupt the MMC pattern in rats. Although porcine motilin reportedly increases gastric emptying in rats (65) the effects of porcine motilin on the rat MMC have not been documented. Furthermore, there is presently no data indicating plasma motilin levels or distribution in the rat using either RIA or ICC. It is evident from several studies that relatively few porcine-motilin antisera cross-react with motilin peptide(s) in rat intestinal extracts. This coupled with the relatively large species difference between canine and porcine motilin, suggests rat motilin may be very different from the porcine peptide.

The overall objective of this work was to further characterize the rat with respect to several aspects concerning the physiology of motilin. Three strategies were applied in working toward this objective. First, porcine motilin was employed in efforts to produce hybridoma cell lines that would secrete monoclonal antibodies with the ability to recognize immunoreactive motilin (IR-M) in rat

intestinal tissue. Second, bipolar electrodes chronically implanted within the rat small intestine were used to record and characterize intestinal interdigestive migrating myoelectric complexes. Third, a combination of the original and more recent techniques for the isolation of motilin were applied to the extraction of motilin like peptide(s) from the rat digestive system. The rat intestinal IR-M obtained was characterized with respect to its chromatographic properties and contractile activity.

METHODS AND MATERIALS

MIGRATING MYOELECTRIC COMPLEX (MMC)

A. ELECTRODE IMPLANT

Wistar rats of either sex weighing between 250-300g were anaesthetized with sodium pentabarbitol, 100µl/100g (Somnotol, M.T.C. Pharmaceuticals; Mississauga, Ontario). A 3cm incision was made in the mid-line of the abdominal wall and the stomach and proximal small intestine exposed. From one to three pairs of bipolar electrodes were attached to the smooth muscle layer at three locations; the antrum, duodenum, and jejunum. Electrodes were constructed from teflon coated stainless steel wire, uninsulated diameter 75 µm. (Mediwire Corp., New York). Teflon insulation was removed from a small segment of the ends to be attached to the smooth muscle. Using a 27 gauge needle as a trocar, electrodes were inserted through the serosa and tied off close to the intestinal wall (Figure 1A). Two such electrodes were inserted approximately 2mm apart to form a bipolar set. Each wire from the three bipolar sets were then bundled together and passed through a puncture wound in the abdominal wall, then tunnelled subcutaneously to the mid-scapular region. Each set was then soldered to a bipolar electrode plug (Plastic Products Company; Roanoke, Virginia) and mounted in dental acrylic. The dental acrylic was moulded to form a flange that could be anchored subcutaneously leaving the electrode plugs exposed. After surgery animals were allowed to recover for 5 days before electrical recordings were made. There was sufficient serosal proliferation around the electrodes during this period to effectively insulate each wire in a bipolar set.

In some animals a jugular cannula was also inserted. This cannula was tunnelled subcutaneously, exposed in the mid-scapular region and anchored to the dental acrylic flange. Glucose was administered orally at a dose of $1 g \cdot kg^{-1}$, and intravenously at a dose of 10 $mg \cdot kg^{-1} \cdot min^{-1}$. Intravenous glucose was administered via a peristaltic pump (Holter, model 921; Pasadena, California) as a sterile solution of 180 mM glucose in 1/3 normal saline (Abbott; Montreal, Quebec) at an infusion rate of approximately 0.1 $ml \cdot min^{-1}$. Cysteamine was administered via the jugular cannula at doses of 6 mg and 11 mg in a 0.25 ml vehicle of sterile saline. Motilin at doses of 5 µg and 35 µg in 0.25 ml sterile saline was also administered via the jugular cannula. Motilin antiserum M03 was diluted 1:1 in sterile saline and 0.50 ml injected into the jugular cannula.

B. ELECTRICAL RECORDING

Following an overnight fast, animals were attached to the recording equipment via the three electrode plugs at the mid-scapular region. Rats were then left unrestrained for the recording period with no access to food or water. Electrical signals from rat smooth muscle were amplified via a Tektronics type 122 low-level preamplifier with a high frequency filtration of 50 Hz, and a time constant of 0.2 sec (Tektronics Inc. Portland, Oregon), and recorded on a Gould model 2200 chart recorder (Gould Inc. Cleveland, Ohio).

MONOCLONAL ANTIBODY PRODUCTION

A. IMMUNIZATION

Natural porcine motilin was conjugated to keyhole lympet haemocyanin (KLH) by the carbodiimide method of Goodfriend (37). The motilin-KLH conjugate was emulsified in complete Freunds adjuvant, and an aliquot equivalent to 50 μg motilin was administered subcutaneously to Balb/c mice. Twenty-one days after the primary injection the mice were bled from a tail vein and tested for antibody production. All animals were then given a secondary injection of motilin-KLH conjugate equivalent to 50 μg motilin. Twelve days after the secondary immunization animals were tested for antibody production. Those animals responding adequately were then rested for thirty days before receiving the final antigen boost prior to fusion. On days 5, 4, and 3 prior to the planned fusion date, immune mice were given a further intravenous injection of 10 μg unconjugated motilin in 0.1 ml saline.

B. PREPARATION OF THE MYELOMA CELL LINE

The NS1 cell line (p3-NS1-Ag4-1; Salk Institute; San Diego, California) was rendered 8-azaguanine resistant by continuous culture in the presence of this drug. The 8-azaguanine resistant cells selected for fusion were stored at -70^0 C in 10% dimethylsulphoxide (DMSO), 20% foetal calf serum (FCS), and 70% Delbecco's Modified Eagle Medium (DME). Several days prior to fusion the NS1 cells were thawed and cultured in DME + 10% FCS. Only NS1 cells in the logarithmic growth phase and at a cell density of approximately 1-2 x 10^5 cells/ml were used for fusion.

C. FUSION AND HAT SELECTION

A motilin immunized Balb/c mouse was sacrificed by cervical dislocation, its spleen was then excised and dispersed into single cell suspension by gentle sieving. Both the spleen and NS1 cells were washed 3 times in warm phosphate-buffered saline (PBS) and resuspended in warm DME. An aliquot of spleen cells were then diluted with a fixed volume of tris-NH_4Cl solution (pH 7.2) to facilitate the lysis of red blood cells. The density of both the NS1 and spleen cells was calculated. They were then mixed at a ratio of 2:1 (spleen:NS1) and spun together at 400 x g for 10 min. The supernatant was removed and the pellet left to equilibrate in a 37^0C water bath for 2-3 min. Following equilibration, 1.0 ml of 50% polyethylene glycol (PEG) (Serva, 33136, M.W. 4000, Heidelburg, W. Germany), in warm DME was added over a period of 1 min with gentle stirring. This was followed by an additional minute of stirring, and the addition of 9.0 ml of warm DME, again with gentle stirring, over a period of 4 min. The fusion mixture was then centrifuged at 400 x g for 10 min and the supernatant discarded. DME was added and the cells incubated at 37^0C for 30 min. During this period a fresh thymocyte-HAT medium was prepared as follows: 20 ml FCS, 1.0 ml 100x hypoxanthine thymidine (HT) buffer plus 0.1 ml 1000x aminopterine (59), single cell suspension or 4 thymuses from 2-4 wk old mice, and DME to 100 ml. The hybrid cells were then gently resuspended in 100 ml thymocyte-HAT medium for every 1 x 10^8 spleen cells used in the fusion. Finally, the cells were gently dispersed into microtitre plates at 0.2 ml per well (2 x 10^5 cells/well), and incubated at 37^0C with 10% CO_2 for approximately 14 days.

D. CLONAL EXPANSION OF HYBRIDOMAS

Hybridoma cultures that exhibited a strong positive reaction on the ELISA were cloned by limiting dilution. Hybrid cultures to be cloned were counted for viable cells using a Speirs-Levy Eosinyl counting chamber. A thymocyte medium was prepared (1 juvenile murine thymus per 25 ml DME + 20% FCS) and used to dilute the desired cultures to a density of 400 cells/ml. Hybridoma cells (at 400 cells/ml) were then serially diluted in six steps, on a 96 well microtitre plate, from 20 cells/well to less than 1 cell/well. Cells were incubated for 8-12 days at $37^{0}C$, 10% CO_2 humidified. When cell cultures reached an appropriate size (individual populations approximately 2mm in diameter; approximately 1-5 x 10^5 cells) their supernatants were tested for motilin antibodies using the ELISA and immunocytochemistry. For ease of handling, hybridomas that tested positive for anti-motilin activity by ELISA were pooled into groups of 2 or 3, then further cultured in 24 well plates for approximately 7 days. Using this method the supernatants from 101 "ELISA positive" cell populations were collected and immunocytochemically screened for anti-motilin activity on rat intestinal tissue.

RADIOIMMUNOASSAY

i) Iodination

Motilin was iodinated following the chloramine-T method of Hunter and Greenwood (47). The ^{125}I-motilin was separated from free ^{125}I on a Sephadex G-25 column (10 x 1 cm) eluted with 0.2M acetic acid, 0.5% bovine serum albumin (BSA), 0.1% trifluoroacetic acid, 1.0% trasylol. Possible fragmentation of ^{125}I-motilin by the iodination procedure was

assessed by the ability of ^{125}I-motilin to bind to dextran coated charcoal. The fraction with the highest percentage of counts (greater than 95%) bound to the charcoal pellet was retained for use in RIA. This fraction was then diluted to approximately $2x10^6$ cpm in acidified ethanol and stored at -20^0 C. When stored in this manner ^{125}I-motilin could be used for up to 10 weeks. Before use in an assay each new batch of ^{125}I-motilin was tested in a standard curve for its ability to be displaced by the addition of natural porcine motilin.

ii) Assay Conditions

Antiserum 13-3* (rabbit; final dilution 1:500,000) or 72-X** (guinea pig; final dilution 1:40,000) was incubated together with ^{125}I-motilin (2000 cpm), sample or standard in assay buffer (0.04M phosphate pH. 6.5, 5% charcoal extracted plasma, 0.25% trasylol) in a final volume of 1.0 ml for 48 h at 4^0 C. Intra-assay and inter-assay variability were obtained from control samples of 2.0 ng/ml which were included in each assay. Assays were accepted when these control values assayed between 1.90 to 2.10 ng/ml motilin.

iii) Separation

Following incubation, bound and free ^{125}I-motilin were separated by adsorption of the free component on dextran coated charcoal. Two hundred microliters of separation buffer prepared as follows: 0.04M phosphate pH 6.5, 5% charcoal extracted plasma, 1.25% charcoal (Fisher Scientific, Vancouver, B.C.), 0.25% dextran M.W. 70,000 (Pharmacia;

---

* Dr. William Chey

** Dr. John C. Brown

Dorval, Quebec), was added to each tube. Tubes were then vortexed and centrifuged at 2000 x g for 30 min. Supernatants were discarded and the tubes allowed to dry inverted for 6 h before counting the pellets. Pellets were counted for 3 min on an automatic gamma counter (Model 1285:Searle Analytic Inc.; Des Plains, Illinois)

CYANOGEN BROMIDE CLEAVAGE

Motilin antisera were characterized with respect to their ability to bind either N-terminal or C-terminal fragments of natural porcine motilin. Amino and carboxyl terminal fragments of motilin were created by cleavage at the methionyl residue with cyanogen bromide (CNBr) (40,41). Cyanogen bromide (Eastman Kodak; Rochester, N.Y.) was dissolved in 70% (v/v) formic acid to a final concentration of 10 mg/ml. Cleavage was performed at 22^{0}C for 6 h in the dark at a peptide concentration of 2mg/ml. Following the 6 h incubation the reaction mixture was diluted 20x with distilled water and lyophilized.

The CNBr cleavage fragments were separated by high pressure liquid chromatography, and identified by both absorption spectra and N-terminal residue determination.

HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC).

A. HPLC EQUIPMENT AND BUFFERS

All HPLC equipment was from Waters Associates Inc. . Reverse phase chromatography was carried out using a μ-Bondapak column (Waters) (3.9mm x 30 cm) consisting of a silica matrix with attached C-18 chains. The solvent system utilized was water and acetonitrile (CH_3CN). Prior to use, each solvent was degassed under vacuum for 10 min., then trifluoroacetic acid added to a final concentration of 0.1%. To prevent reabsorption of atmospheric gases, solvents were also maintained in an atmosphere of helium during their use. Samples were applied via a Waters U6K injection system, and mixing of the solvents to form gradients of decreasing polarity was accomplished by using a Waters 660 solvent programmer. Effluent was monitored for absorbance at 220 nm.

B. ELUTION WITH ACETONITRILE

A semi-empirical approach was applied to the elution of compounds from the μ-Bondapak column with acetonitrile. Only linear gradients were used and generally a wide gradient (5%-75% acetonitrile) was applied over 30 min in a preliminary run to gain an estimation of the concentration range in which peptides in the mixture eluted. Upon determining the acetonitrile concentration range in which the sample eluted it was possible to progressively raise the initial acetonitrile concentrations and lower the final acetonitrile concentrations of the gradient in order to optimise resolution and elution time. The time

over which the gradient was run was also progressively shortened so that an optimal gradient slope was maintained (generally less than a 3% rise in acetonitrile concentration per min).

Sensitivity of the detection apparatus also required adjustment according to the size and concentration of the sample being chromatographed. Monitoring the effluent for absorbance at a wavelengh of 220 nm provided the greatest sensitivity for peak detection. When concentrated samples of a relatively crude extract were chromatographed the sensitivity of the spectrophotometer was reduced to 2.0 Arbitrary Units Full Scale (AUFS). As well, the chart recorder could be adjusted to attenuate peaks in order that relatively large amounts of protein did not exceed the limit of detection. Similarly the sensitivity of the spectrophotometer was increased (to a maximum of 0.01 AUFS) and attenuation of recorded peaks reduced when small relatively pure samples were chromatographed.

Spectrophotometric detection and recording of protein peaks was simultaneous with column outflow. This facilitated the separation and collection of peaks by pooling of the outflow during each spectrophotometric peak and discarding the effluent between peaks.

In order to check the homogeneity of collected peaks, they were first lyophilized, reconstituted in dH_2O: 0.1% TFA, then rechromatographed on HPLC.

IDENTIFICATION OF MOTILIN FRAGMENTS

A) Spectrophotometric analysis

Peaks identified from the HPLC chromatographs of CNBr cleaved material were subjected to spectrophotometric analyses in the

ultraviolet range using a Pye Unicam Sp8-100 spectrophotometer. As motilin contains a single tyrosine residue on the N-terminal side of the CNBr cleavage site, only uncleaved motilin and the N-terminal fragment are expected to exhibit an absorption peak at 280 nm.

B) N-terminal residue determination

Amino terminal residues of CNBr cleaved motilin fragments were determined using the dansyl (DNS) method of Gray (38). Dansyl-amino acids were detected by the method of Woods and Wang (102) in which thin layer chromatography on 5 cm^2 polyamide plates is employed.

Approximately 5nMol of uncleaved motilin or CNBr cleaved motilin fragments were aliquoted into 6 x 50 mm glass culture tubes and lyophilized. Samples were then dissolved in 2.0 ul of 1% sodium bicarbonate, centrifuged and relyophilized. Samples were redissolved in 2.5µl distilled water, and 2.5µl of dansyl-chloride (5-dimethylaminonapthalene-1-sulphonyl chloride, 2.5mg/ml in acetone; Sigma, St Louis, Missouri.) added. This reaction mixture was then centrifuged, sealed and incubated for 20 min at 45 ^{0} C., following which the tubes were again centrifuged and relyophilized. Dansyl-peptides were then acid hydrolized by adding 50 µl of 5.7M HCl, centrifuging, sealing the tube in a flame and heating at 100^0 C. for 18 h.

Following acid hydrolysis the tubes were centrifuged, opened, diluted 50% with distilled water and lyophilized over sodium hydroxide pellets. Thin layer chromatography on polyamide plates (Chen Chin trading Co. Taipai, Taiwan) was employed to identify dansyl-amino acids. To each tube 2.5 µl of 50% pyridine was added, and 0.25 µl

spotted on each side of a 5 cm^2 polyamide plate. A standard solution containing DNS-glutamic acid, DNS-phenylalanine, DNS-Ɛ-lysine, DNS-tyrosine and DNS-amide each at approximately 0.5mg/ml was also spotted onto one side of the plate. Ascending chromatography was performed in two dimensions with the following solvents:

Dimension	Solvent	
1	I	water/90% formic acid (200/3 v/v)
2	II	benzene/acetic acid (9/1 v/v)
2	III	hexane/butanol/acetic acid (3/3/1 v/v/v)

Plates were viewed under a short wave ultraviolet source, with identification being made by comparison to the standards on one side of the plate.

ENZYME-LINKED-IMMUNOSORBENT-ASSAY (ELISA)

Carboxy-terminal (1-13) and amide-terminal (14-22) motilin fragments generated by CNBr cleavage were utilized in the ELISA as a means of identifying the binding epitopes of motilin antisera. A 96 well microtitre plate (Falcon) was initially coated with motilin. The coating procedure consisted of dissolving 0.5 µg motilin per ml carbonate buffer (15mM sodium carbonate, 35mM sodium bicarbonate, 3mM sodium azide, pH 9.6) and dispensing 100µl to each well. Plates were then incubated at 4^0 C for 24 h. The motilin antisera were applied after washing the plate 3X with PBS-Tween. After application of the first antibody layer, plates were incubated at 22^0 C for 2 h. The plates were again washed with PBS-Tween and the second antibody

(rabbit anti-mouse at 1:3000 or goat anti-rabbit at 1:2000 or goat anti-guinea pig at 1:2000) covalently linked to alkaline phosphatase was added (100µl) to each well. The plates were again incubated at room temp. for 2 h, and subsequently washed 3X with PBS-tween. The assay was developed by adding 100 µl of 1mg para-nitro-phenyl-phosphate(Sigma; St Louis, Missouri) in 1ml of diethanolamine buffer (10% diethanolamine, 0.5mM magnesium chloride, 3mM sodium azide) and the phosphatase reaction was monitored at 405 nm on a Micro ELISA reader (MR 580, Dynatech; Alexandria, Virginia). To determine the regional specificity of the motilin antisera an inhibition ELISA was performed. In this instance motilin antisera (first antibody layer) were incubated at 4^0 C for 24 h with either uncleaved motilin, the C-terminal fragment, the N-terminal motilin fragment, or no peptide. All other aspects of the ELISA were as described above.

IMMUNOCYTOCHEMISTRY

i) TISSUE FIXATION

Antisera were also tested immunocytochemically on sections of rat duodenum, ileum, jejunum, cerebellum, and porcine duodenum. Three methods of fixation were used for rat tissues.

A). Bouins

Fresh samples of rat duodenum, ileum, and jejunum were rinsed with PBS and immediately placed in Bouins fixative (75% picric acid, 25% v/v of a 37% solution of formaldehyde in distilled water, and 3% glacial acetic acid) for up to 18 h. After fixing in Bouins the tissue was dehydrated and embedded in paraffin using a tissue processor

(Fisher Histomatic; model 166). Bouins fixed tissues were sectioned at 5μm and mounted on uncoated glass slides by heating to 37^0 C overnight.

B). Benzoquinone fixation

Rats anaesthetized with urethane were perfused through the descending aorta with PBS until relatively free of red blood cells (approximately 100ml). Immediately following perfusion with PBS, animals were fixed by perfusing with 250ml of a freshly prepared solution of 0.4% benzoquinone in PBS, pH 7.2-7.4. The benzoquinone solution was shielded from light, and perfusion pressure was approximately 150 cm H_2O. After perfusion tissues were removed and post-fixed in the benzoquinone solution for 2 h. Excess benzoquinone was removed by soaking in several changes of PBS + 5% sucrose. Tissues were stored in PBS + 5% sucrose until sectioned.

C). Paraformaldehyde

Whole rats were fixed (as described for benzoquinone) with a 4% solution of paraformaldehyde in 0.1M phosphate, 1mM Mg^{++}. Tissue was postfixed overnight in paraformaldehyde, and stored in 5% sucrose as above. Benzoquinone and paraformaldehyde fixed tissues were cut at 10μm on a cryostat and mounted on formol-gelatine coated slides (intestine), or suspended in PBS, 0.2% azide (cerebellum).

ii) STAINING

Tissue sections either mounted on slides (small intestine) or free floating (cerebellum) were incubated for 24 h at 4^0 C with motilin

antisera in dilutions ranging from 1:100 to 1:5000. Antisera used in staining Bouins fixed material were diluted in PBS with 1% BSA. Antisera used in staining benzoquinone or paraformaldehyde fixed tissues were diluted in 50mM Tris with 0.3 % Triton x-100. Hybridoma supernatants were immunocytochemically screened neat and at dilutions of 1:10 and 1:50 in PBS with 1% BSA. Following the initial incubation, slides were washed 3 times for 5 min periods in PBS, and then incubated for 1 h with fluorescein labelled goat antiserum to rabbit IgG (Calbiochem; La Jolla, California) or to fluorescein labelled rabbit anti-guinea pig IgG (Miles-Yeda; Naperville, Illinois) diluted 1:200 in PBS (the host species of the primary antisera are indicated in table 1). Sections were then washed 3 times as above and subsequently mounted in glycerine PBS (3:1). Sections were examined with a fluorescence microscope under ultraviolet light (220).

Alternatively a peroxidase immunostain was used in conjunction with guinea pig antiserum, or a peroxidase-antiperoxidase stain was used with rabbit antiserum. Where the first layer consisted of a guinea pig derived antiserum, a peroxidase conjugated rabbit anti-guinea pig serum (Dako; Santa Barbara, California) at 1:200 was applied as the second layer and incubated for 30 min. The stain was then developed by immersing the slides in PBS buffer pH 7.3; containing 0.01% hydrogen peroxide and 0.05% 3,3'diaminobenzidine tetrahydrochloride (DAB) (BDH chemicals; Toronto, Ontario). Where the first layer consisted of a rabbit derived antiserum (ie: 13-3) a goat anti-rabbit serum (Calbiochem; La Jolla, California) was applied at 1:100 and incubated for 30 min. The second layer was then thoroughly washed off and a rabbit peroxidase anti-peroxidase complex (Dako;

Santa Barbara, California) applied at 1:200. Slides were then developed as above and counterstained with haematoxylin.

TISSUE EXTRACTION

Wistar rats were anaesthetized with pentobarbitol; 100µl/100g, and the proximal 40-50 cm of small intestine removed and immediately frozen in liquid nitrogen. These tissues were then either immediately used or stored at -70^0 C for later extraction. Frozen tissue was first boiled for 10 min in an equal volume (w/w) of distilled water, allowed to cool then homogenized for 2 min (Tekmar Tissumizer; Cincinnati, Ohio). After homogenization an equal volume of 4% trifluoroacetic acid was added, and the suspension left stirring at 4^0 C for 12 h. The acid extract was then centrifuged at 15,000 x g for 90min, the supernatant precipitated with NaCl (30g/100ml), and the tissue pellet re-extracted as above. The salt pellet was collected by centrifugation and extracted 2x in methanol pH 6.0 (25ml/g). Methanol soluble material was precipitated with 2 volumes cold ether and collected by centrifugation. The ether precipitate was dried under a stream of N_2 and then dissolved in 0.1% TFA to a concentration of 15mg/ml. The dissolved ether precipitate was then loaded in 50 ml aliquots onto Sep-Pak C_{18} cartridges (Waters Associates; Mississauga, Ontario) using a syringe driven by a Harvard pump at a flow rate of approximately 1.0ml/min. After loading, the cartridges were washed with 5ml 0.1% TFA followed by 5 ml of 5% acetonitrile, 0.1% TFA. Cartridges were then eluted with 2ml 50% acetonitrile in 0.1% TFA. Acetonitrile was eliminated from the 50% eluate by evaporation under a stream of N_2.

CHROMATOGRAPHIC CHARACTERIZATION OF RAT INTESTINAL IR-M

A) ION EXCHANGE CHROMATOGRAPHY

The Sep-Pak concentrated extract was dialyzed overnight in 0.02M ammonium acetate adjusted to pH 5.5 with dilute acetic acid. The dialyzed concentrate was then loaded onto a SP-Sephadex-C25 column (1.5x30 cm) and eluted with 150ml of 0.02M ammonium acetate pH 5.5. The column was then eluted with a further 110ml of 0.3M ammonium acetate, adjusted to pH 8.0 with 0.02M ammonia. Flow rate was approximately 1.5ml/min. Fractions (3ml) were monitored for absorbance at 280 nm and assayed for IR-M by RIA.

B) GEL FILTRATION CHROMATOGRAPHY

The SP-Sephadex-C25 fractions containing the IR-M were pooled and applied to a Biogel P-10 (200-400 mesh) gel filtration column (2.5 x 20 cm). The column was eluted with 0.2M acetic acid at a flow rate of 0.6 ml/min. Fractions (3ml) were collected and monitored for absorbance at 280 nm and assayed for IR-M by RIA.

C) HPLC

The IR-M peak obtained from the gel filtration step was pooled and lyophilized. The lyophilized material was reconstituted with 0.1% TFA and applied to a 3.9mm x 30cm μ-Bondapak column (Waters; Mississauga, Ontario). The column was eluted at a flow rate of 1.5 ml/min and developed with a linear gradient of water/acetonitrile; 0.1% TFA 15-45% over 30 min. Fractions were collected every 0.5 min and assayed for IR-M by RIA.

Porcine motilin submitted to an extraction protocol similar to that of rat intestinal tissue was chromatographed as an external and internal standard for comparison to rat intestinal IR-M.

SMOOTH MUSCLE CONTRACTILE ACTIVITY

New Zealand White rabbits of either sex were anaesthetized with a solution of 30% urethane 1% alpha-chloralose i.v., and the duodenum and the proximal 5cm of jejunum removed rapidly and placed in Krebs' ringer bicarbonate at 22^0C. The solution (in mM) was composed of KCl 4.4, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.5, $NaHCO_3$ 25, NaCl 120, dextrose 9.0. Choline chloride was added to a final concentration of 10 µM and the solution maintained at 37^0 C and gassed with 95% O_2, 5% CO_2. Longitudinal segments of duodenum in lengths of 1.5-2.0cm were suspended vertically in a 5 ml organ chamber containing Krebs ringer bicarbonate. The upper end of the strips was connected to a force-voltage transducer (Grass model TF03; Quincy, Massachusetts) and the signal amplified (Grass model 7DA) and recorded on a chart recorder (Grass model 79WU). Tissues were suspended and adjusted to a tension of approximately 1g, then allowed to equilibrate for 15 min. Following the initial equilibration, porcine motilin at 10^{-7}M was applied and rinsed out as soon as a maximal response was seen. The preparation was then allowed to equilibrate for a further 20-30 min. After final equilibration the preparation was tested for desensitization to porcine motilin by administering a subsequent dose of motilin within 4-5 min of a previous dose. The response to the second dose was then compared to that of the first. In each experiment of this type a dose of acetylcholine was given both before and after motilin

administration as a means of gauging the sensitivity of the muscle strip over the experimental period. In a similar experiment rat intestinal extract was administered to the muscle bath within 4-5 min of a previous dose of porcine motilin, and the contractile response compared to that induced by the extract without prior administration of motilin.

RESULTS

MIGRATING MYOELECTRIC COMPLEX

Regular patterns of myoelectric activity in the intestine of the fasted rat have been recorded from chronically implanted bipolar electrodes. Polyphasic electrical complexes with a frequency of 4-5/sec (fig 1C) occurred superimposed on a background of regular and continuous slow waves with a frequency ranging from 40-43/min (fig.1B). The polyphasic electrical complexes occurred in cyclic association with the slow waves and were represented by 3 recognizable phases. 1) A 4-5 min period of almost complete absence of spike bursts followed by, 2) a 1-1.5 min period of irregular spike burst occurring at a frequency ranging from 6-24/min. 3) The period of irregular spiking activity was followed by a 2-3 min period of intense spiking activity during which spike bursts occurred with every slow wave (fig.1D). To improve the recognition of myoelectric patterns recorded the number of spike bursts per 20 sec interval was summed and plotted as "integrated activity". The duodenal myoelectrical pattern exhibited by fed rats (fig 2A) consisted of irregular spike bursts occurring with an average frequency of 15-18/min. The integrated myoelectrical pattern in the fed rat was noticeably different from that seen in the fasted rat (fig 2B)

Intravenous (i.v.) glucose administered at a dose of $10 \cdot mg \cdot kg^{-1} \cdot min^{-1}$ via a cannula into the jugular vein had no effect on the period or intensity of myoelectric complexes recorded from the duodenum of fasted rats (fig 3A). A single oral glucose dose of $1g \cdot kg^{-1}$, however, rapidly resulted in the disruption of the fasted pattern of myoelectric activity and the appearance of continuous spike bursts at

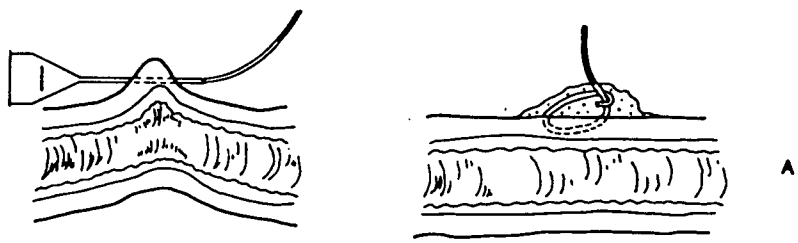
submaximal frequency (fig. 3B), a pattern characteristic of the fed state.

Intravenous administration of 6 mg cysteamine in 0.25 ml saline had no effect on the fasted pattern of periodic activity. Administration of 11 mg cysteamine in 0.25 ml saline, however, prolonged the third phase of periodic activity (spike bursts at maximal intensity) so that the period of quiescence between complexes was abolished (fig. 2B).

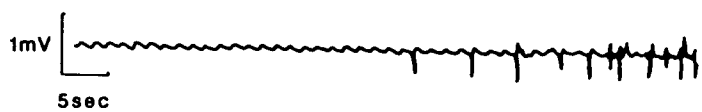
Intravenous administration of 5 µg motilin in 0.25 ml saline and 35 µg motilin in 0.25 ml saline did not affect the fasted pattern of periodic activity recorded from the duodenum of the fasted rat. Similarly, administration of 500 µl motilin antiserum M03 diluted 1:1 in saline had no affect on the fasted pattern of myoelectric activity (fig 4).

HYBRIDOMA PRODUCTION

In general Balb/c mice showed a moderate to strong immune response to subcutaneous injection of motilin-KLH conjugate in complete Freunds adjuvant. This is depicted by the high anti-motilin titre seen in mouse serum 28 days after primary injection of motilin-KLH conjugate (50 µg motilin equivalent) (fig 5). Fusions were also successful, resulting in greater than 50% of the primary fusion colonies with a positive reaction to porcine motilin upon screening with ELISA. However, subsequent ICC screening of approximately 101 "ELISA-positive" clones on sections of rat duodenum and ileum, produced only negative results.

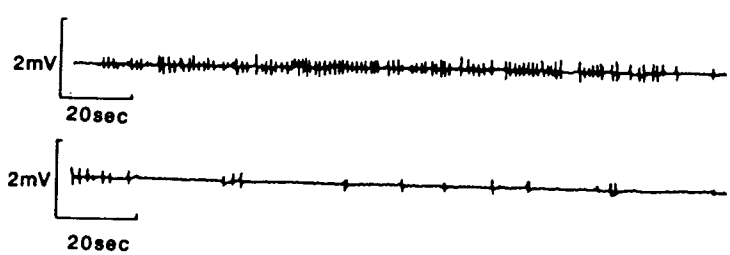


A



B

C



D

Figure 1. A) Diagramatic illustration of electrode insertion into the intestinal smooth muscle. A 27 gauge needle was used as a trocar to insert stainless steel wire (75 μm in diameter) through the muscle layer. The wire was tied off close to the intestinal wall, and proliferation of serosal tissue achieved fixation and insulation within 5 days. B) Electromyogram (expanded scale) showing slow waves with a frequency of 42/min. C) Expanded scale showing polyphasic spike burst with a frequency of approximately 4-5/sec. D) Electromyogram (reduced scale) showing 6 min continuous recording from duodenal bipolar electrode. Two phases of activity are depicted, a 2.4 min period of spike burst with every slow wave, followed by approximately 3 minutes of quiescence.

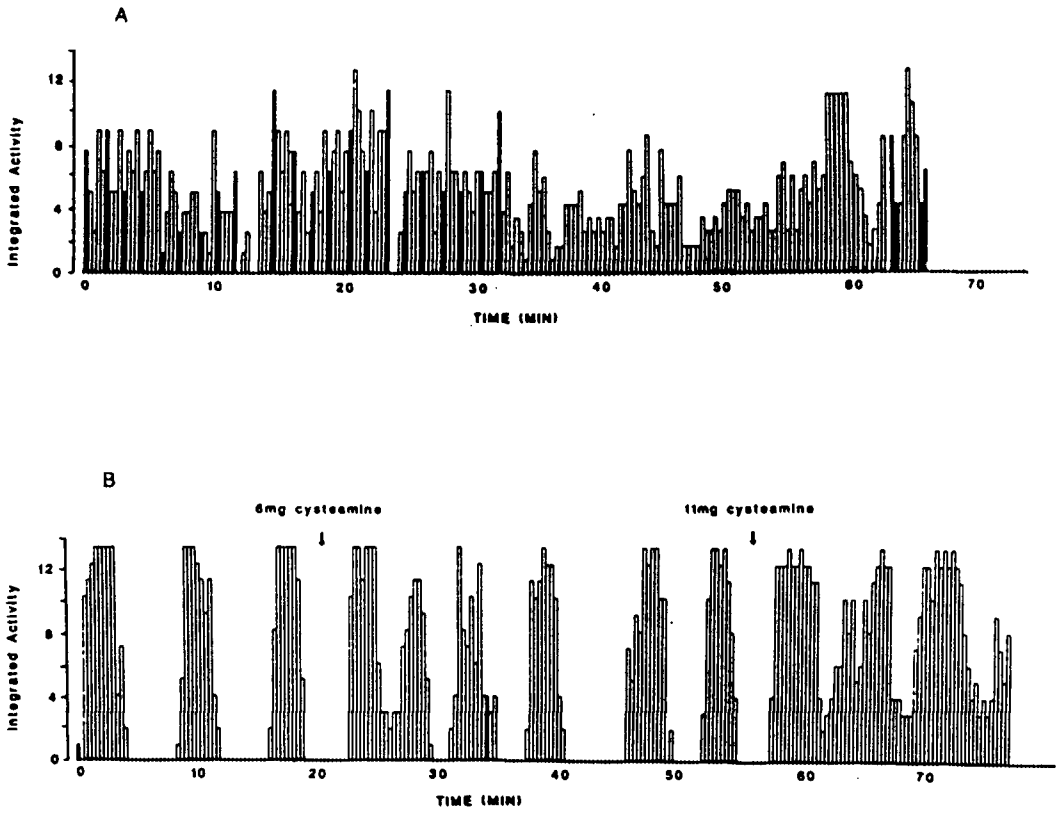


Figure 2. A) Histogram of integrated activity (spike bursts/20 sec interval) recorded from the duodenum of a fed rat. The frequency of spike bursts are submaximal and display no distinct pattern. Representative of 3 trials. B) Histogram of integrated activity recorded from the duodenum of a fasted rat. Spike bursts occur at maximal frequency for periods of 2-3 min, followed by 6-7 min of quiescence and a return to maximal activity. 6 mg cysteamine (i.v. in 200µl saline) had no effect on this pattern, but 11 mg cysteamine disrupted the pattern by eliminating the period of quiescence. Representative of 2 trials.

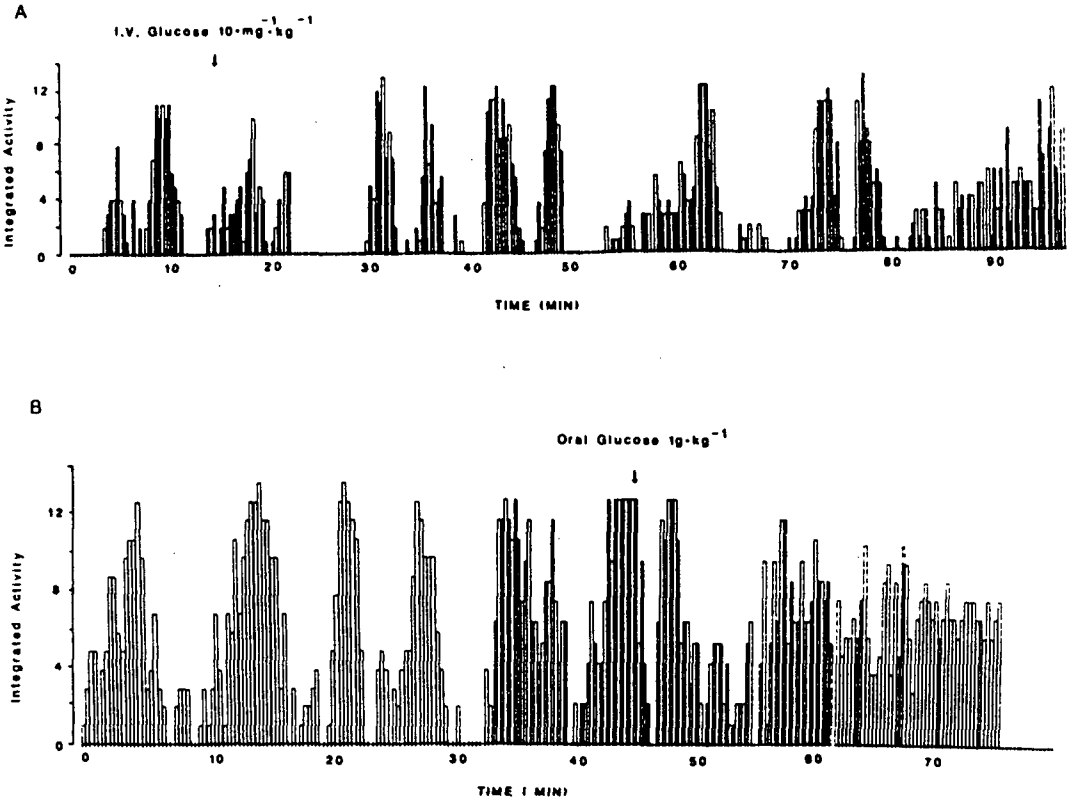


Figure 3. A) Histogram depicting integrated activity (spike bursts/20sec interval) recorded from the duodenum of a fasted rat. The administration of 10 $mg \cdot kg^{-1} \cdot min^{-1}$ glucose i.v. had no effect on the myoelectric pattern. Representative of 2 trials. B) Histogram of integrated activity from the duodenum of a fasted rat. The administration of oral glucose ($1g \cdot kg^{-1}$) was followed by a disruption of the fasted pattern of activity and the introduction of a fed pattern of myoelectric activity. Representative of 2 trials.

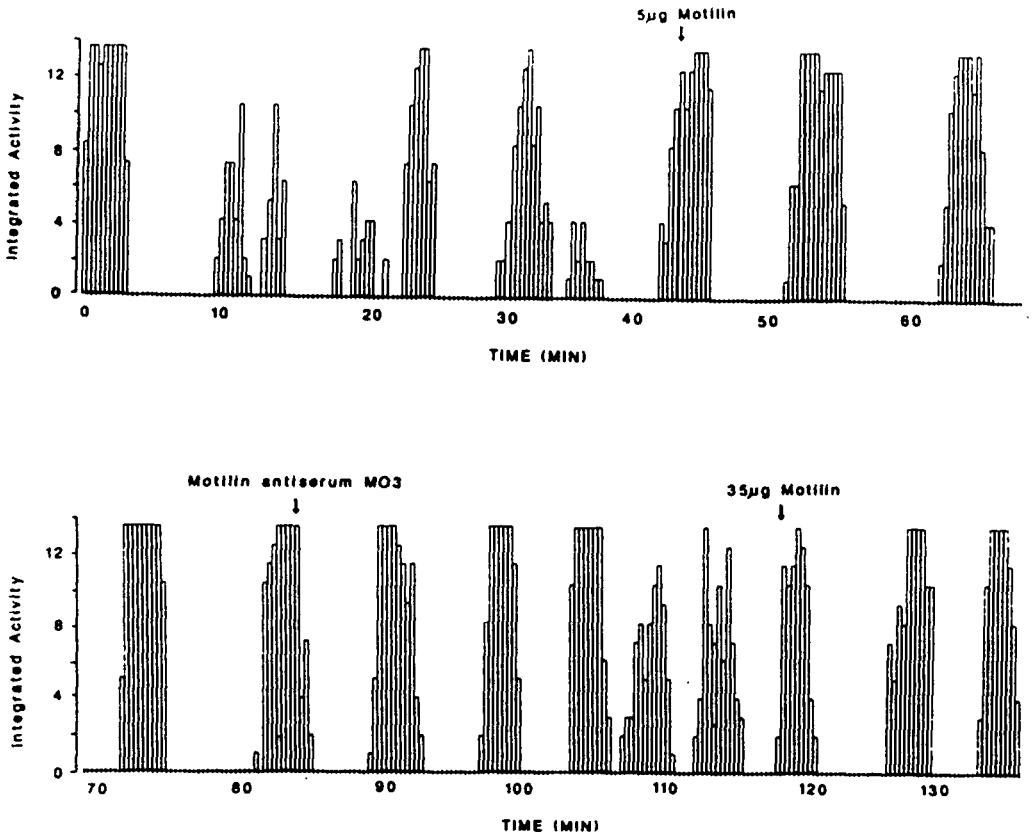


Figure 4. Histogram depicting 135 min continuous recording of integrated activity (spike bursts/20sec interval) in a fasted rat. The intravenous administration of porcine motilin or antiserum directed against porcine motilin had no effect on the myoelectric pattern.

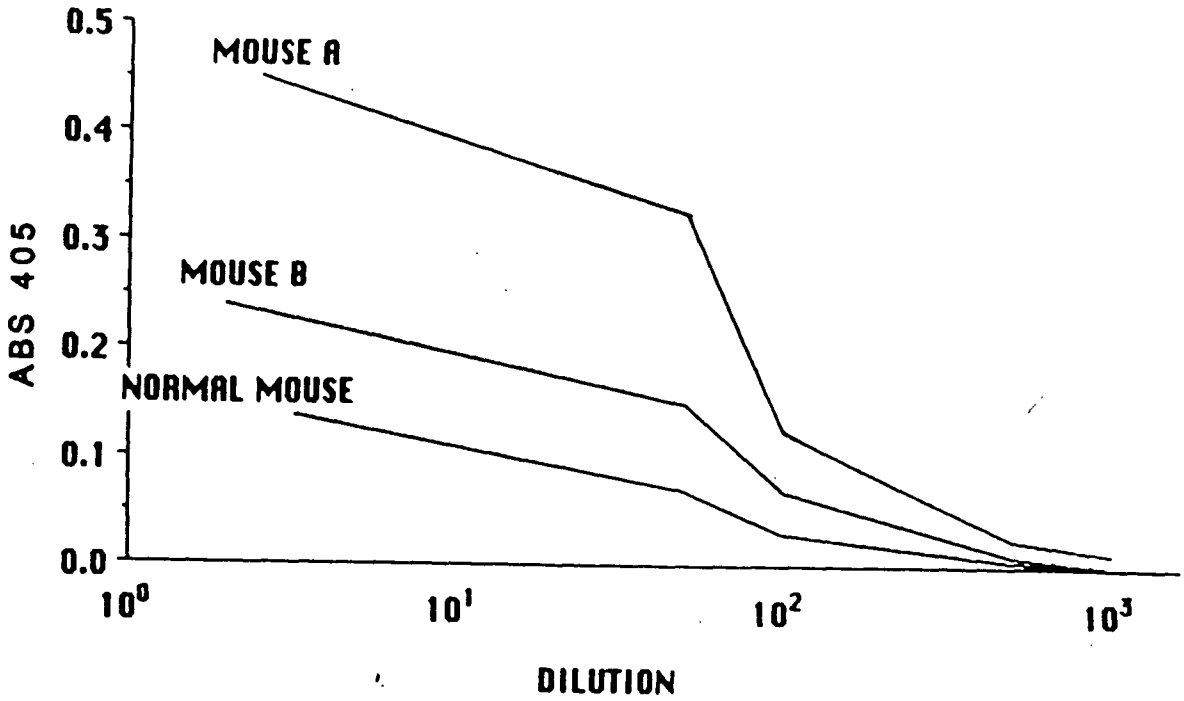


Figure 5. Enzyme linked immunosorbent assay for anti-porcine-motilin activity in serum from 3 mice. Mouse A and B were injected with motilin-KLH congugate (equivalent to 50µg motilin) 21 days prior to assay. Normal mouse was from the same litter but received no motilin. After subsequent injections of motilin-KLH conjugate, spleen cells from mouse A were used for fusion.

CNBr CLEAVAGE OF NATURAL PORCINE MOTILIN

The lyophilized CNBr reaction mixture was redissolved in 500 µl 0.1% TFA and centrifuged. In a typical experiment 100 µl of the resuspended cleavage material was injected into the HPLC system and eluted with a linear gradient of 17% to 57% acetonitrile over 15 min. Six major peaks were identified and labelled A through F (fig. 6A). Application of a CNBr blank (a reaction mixture identical to that used for motilin cleavage but containing no motilin) to the HPLC system and elution in the same manner identified no spurious peaks attributable to impurities in the CNBr (fig.6C). Similarly, addition of 20µg motilin to the CNBr cleavage material showed uncleaved motilin eluting in the approximate position of peak C (fig.6B).

Five separate runs of the cleavage material were made through the HPLC system. Peaks A through F were collected separately for each run. Corresponding peaks for each run were pooled and lypophilized. Each of the pooled peaks were then reconstituted in 200 µl of HPLC water (0.1% TFA) and re-chromatographed on the HPLC system. The re-chromatographed material was isocratically eluted (no change in solvent concentration) at 26% acetonitrile. A representative chromatogram of the three major peaks is shown in figure 2. Isocratic elution of peak E demonstrated a minor contaminant with an elution time of approximately 6 min (fig 7A). This contaminant probably resulted from an overlap in peak collection during the initial chromatograph. The improved resolution with the re-chromatographed material, however, allowed for a more precise peak collection and consequently improved purification of fractions. Isocratic elution of peak B indicated that this fraction was collected relatively free of contamination from adjoining peaks

(fig 7B). Peak F was re-chromatographed both with and without the addition of a 6 μg standard of uncleaved porcine motilin. Under these conditions (26% acetonitrile) motilin eluted at approximately 5.5 min. (fig 7C). Peak F appears to have been contaminated with material from peak E during the initial collection, as isocratic elution reveals two peaks, one with an elution time identical to peak E (fig 7C). All major peaks from isocratically eluted material were collected and lyophilized. Lyophilized material was then reconstituted in 50 mM acetic acid.

IDENTIFICATION OF MOTILIN FRAGMENTS

A.) U.V. Spectra

Ultra violet absorption spectra of peaks A through F revealed that the major tyrosine component was contained in fraction E (fig 8). Thus this fraction was designated as the N-terminal fragment. The only other fraction to show a minor peak at 280 nm was fraction D (not shown). The other two major peaks, B and F, did not exhibit any adsorption peaks at 280 nm. Either peak B or peak F was therefore designated as the C-terminal fragment.

B.) N-Terminal Residue Determination

Cleavage at the methionyl residue with CNBr yields a 13 amino acid N-terminal fragment (CN 1), and a 9 amino acid C-terminal fragment (CN 2) .

CN1 H_2N-Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-Arg-Met-COOH

CN2 H_2N-Gln*-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln-COOH

* Motilin heterogeneity is possibly demonstrated by the presence of glutamic acid at position 14 (88b).

The amino groups available for dansylation in the C-terminal fragment include the alpha-amino of the N-terminal glutamine as well as the epsilon-amino groups of the lysine residues at position 16 and 20. The only amino group available for dansylation in the N-terminal motilin fragment is the alpha-amino of phenylalanine at the N-terminus. The OH group on the aromatic side chain of tyrosine at position 7 also provides a reactive site for dansylation within the N-terminal region 1-13.

DNS-tyrosine and DNS-phenylalanine were the only DNS-amino acids detected for peak E from the HPLC fraction, thus confirming this fraction as the N-terminal fragment (fig 9A). Peak B was the only HPLC fraction yielding epsilon-DNS-lysine. DNS-tyrosine and DNS-phenylalanine were not detected in fraction B, thus confirming this fraction as the C-terminal motilin fragment. Of the minor peaks A, C, D, only peak D showed a slight absorbance peak at 280 nm which, in conjunction with co-elution of motilin at similar times (fig 6B), suggests that peak D represents uncleaved motilin. Dansylation of fraction A, C, and D, however proved inconclusive. Fraction F is suspected as the homoserine-lactone variant of the N-terminal motilin fragment as this fraction demonstrated the presence of low levels of DNS-phenylalanine and DNS-tyrosine. The lack of an absorbance peak at 280 nm for fraction F may simply reflect a low fragment concentration.

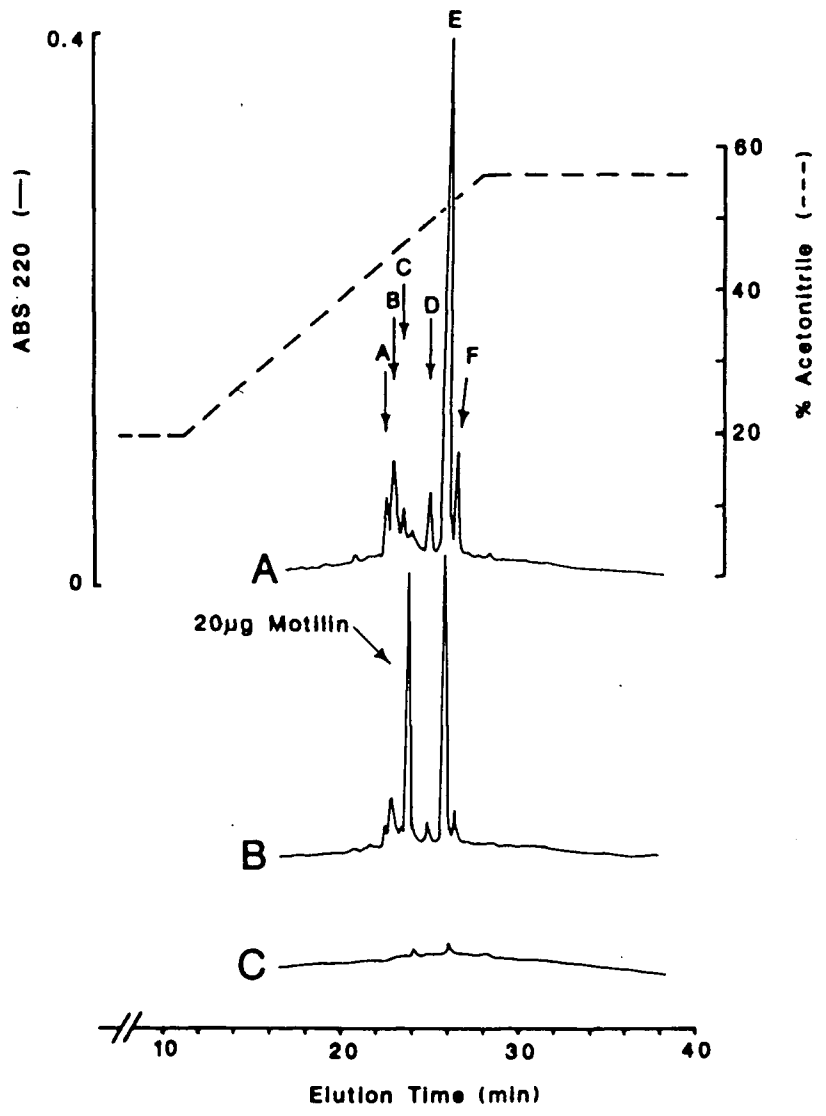


Figure 6. Chromatograms of CNBr cleaved porcine motilin, obtained by reverse phase HPLC with a linear gradient consisting of water/acetonitrile. A) chromatogram of 200µl CNBr cleaved motilin. B) chromatogram of 100µl CNBr cleaved material with addition of 20µg internal motilin standard. C) chromatogram of 100µl of CNBr blank.

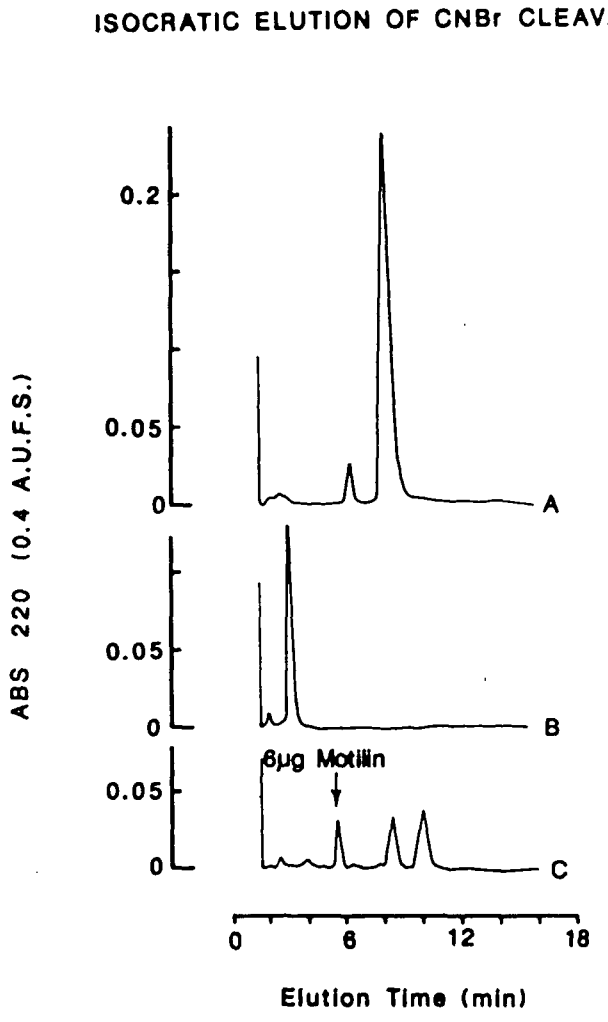


Figure 7. Chromatograms of major pooled peaks from the initial HPLC separation of CNBr cleaved porcine motilin. Chromatograms were obtained by reverse phase HPLC isocratically eluted with 26% acetonitrile. A) chromatogram of peak "E". B) chromatogram of peak "B". C) chromatogram of peak "F" with addition of an internal standard of 6µg porcine motilin.

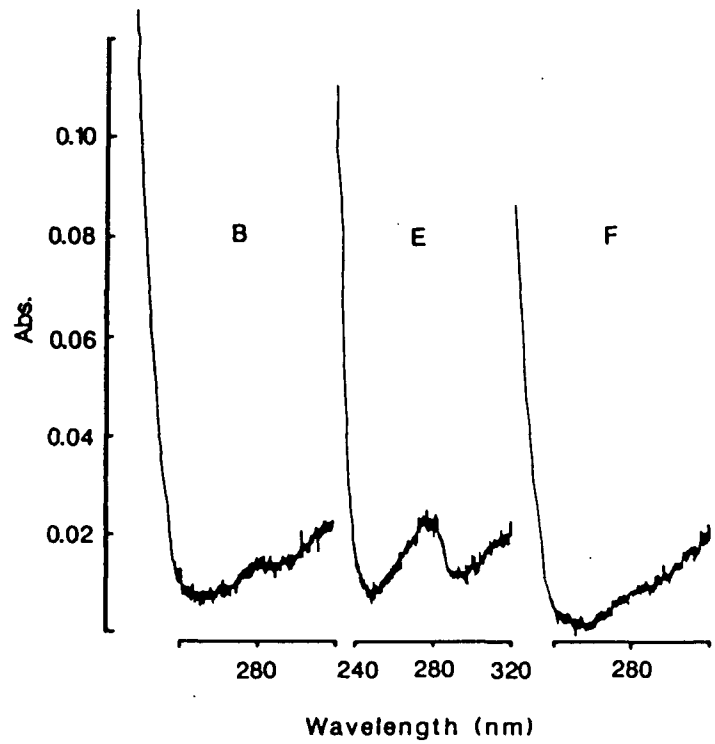


Figure 8. Ultra violet spectra of major peaks from initial HPLC separation of CNBr cleaved porcine motilin. Left) UV spectra of peak "B" . Centre) UV spectra of peak "E", with absorbance peak at 280 nm indicating presence of tyrosine residue. Right) UV spectra of peak "F".

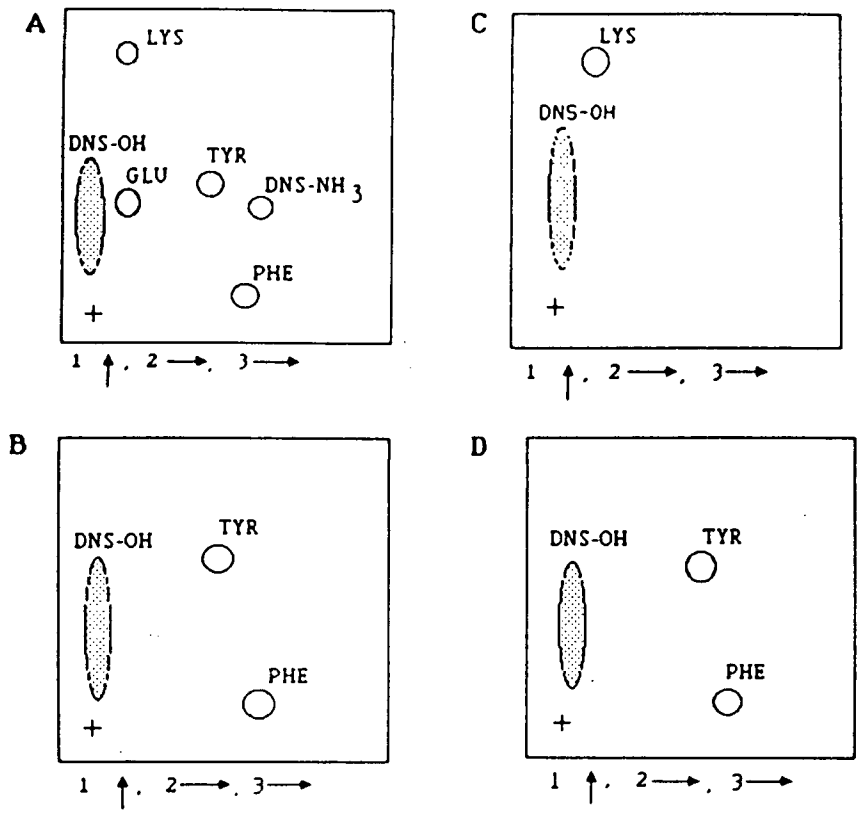


Figure 9. Thin layer chromatography of dansyl amino acids generated from CNBr cleavage fragments of porcine motilin. Chromatograms were obtained by spotting hydrolysed material onto 5x5 cm polyamide plates and running as described in the methods section. A) standard containing DNS-tyrosine, DNS-lysine, DNS-phenylalanine, DNS-glutamic acid and DNS-amide. B) chromatogram of DNS-amino acids from peak "E". C) chromatogram of DNS-amino acids from peak "B". D) chromatogram from peak "F". Solvents and direction of solvent migration is as indicated by arrows.

CHARACTERIZATION OF MOTILIN ANTISERA

Antisera 72x and 74 were produced in guinea pigs by immunization with porcine motilin conjugated to bovine serum albumin (BSA) using the carbodiimide method. Antisera 13-3, Mx, and M03 were produced in rabbits by immunization with porcine motilin conjugated to BSA using the carbodiimide method. Pre-incubation of antisera 72x and 13-3 with the C-terminal fragment of motilin prior to application to the ELISA (fig 10), produced inhibition curves identical to those produced by pre-incubation with uncleaved motilin, indicating that these antisera are highly specific for the C-terminal region of motilin. These antisera also demonstrate a minor component with N-terminal specificity, as pre-incubation with the N-terminal fragment produced a maximal inhibition of between 10-20%. Antisera 74 and M03 also demonstrate a primary specificity directed toward the C-terminal region of motilin, however, a relatively high proportion of the antibodies in these antisera are also capable of detecting the N-terminal region of motilin (fig 11).

RADIOIMMUNOASSAY

Three different antisera were used for RIA's: 13-3, 72X, and M03. The other antisera or monoclonal antibodies listed in table I were not appropriate for RIA. Of the three antisera used, 13-3 and 72X were able to detect IR-M peptides in the Sep-Pak concentrated extract of rat intestinal tissue (Fig 12) Both antisera 13-3 and 72X demonstrated similar binding charateristics for IR-M in rat intestinal extracts, as serial dilution of this extract produced curves parallel to the standard curve. Antisera 13-3 demonstrated considerably higher titre than antisera 72x, 13-3 was therefore employed in the RIA used to monitor the extraction procedures.

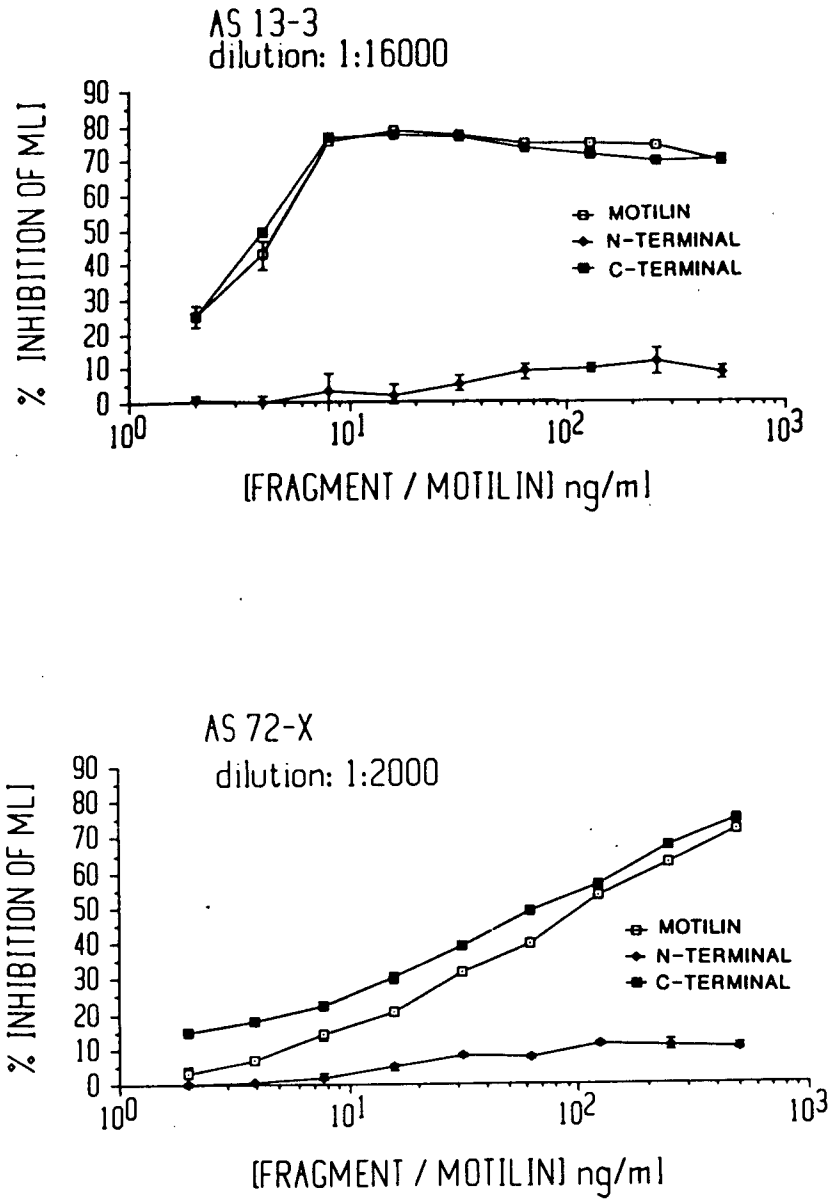
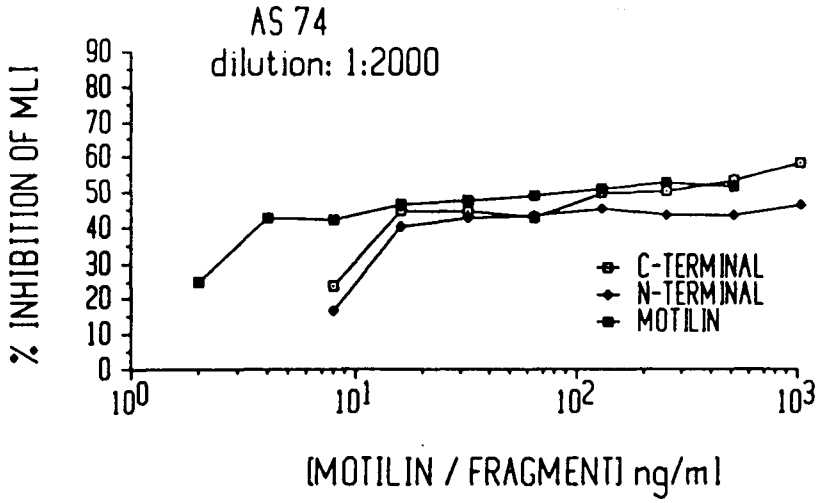


Figure 10. Regional specificity of antisera 72X, and 13-3 as demonstrated with the Enzyme Linked Immunosorbent Assay. Each antiserum was pre-incubated for 24 h at 4^{0} C with either natural porcine motilin, N-terminal fragment 1-13, C-terminal fragment 14-22, or no peptide. The antisera were then applied to motilin coated micro-titre plates and the assay developed as described in the methods. Inhibition of immunoreactivity was calculated as a percentage of phosphatase activity in wells containing motilin antisera that had not been pre-incubated with motilin or motilin fragments.



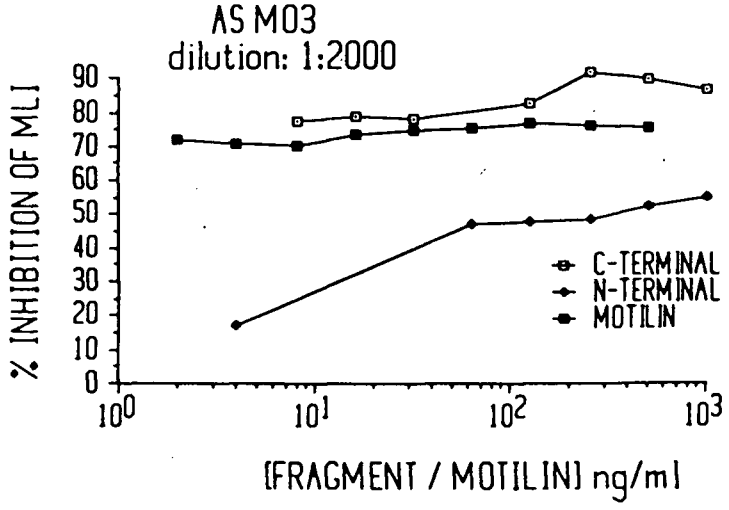


Figure 11. Regional specificity of antisera M03, and 74 as demonstrated with the Enzyme Linked Immunosorbent Assay. Each antiserum was pre-incubated for 24 h at 4^0 C with either natural porcine motilin, N-terminal fragment 1-13, C-terminal fragment 14-22, or no peptide. The antisera were then applied to motilin coated microtitre plates and the assay developed as described in the methods. Inhibition of immunoreactivity was calculated as a percentage of phosphatase activity in wells containing motilin antiserum that had not been pre-incubated with motilin or motilin fragments.

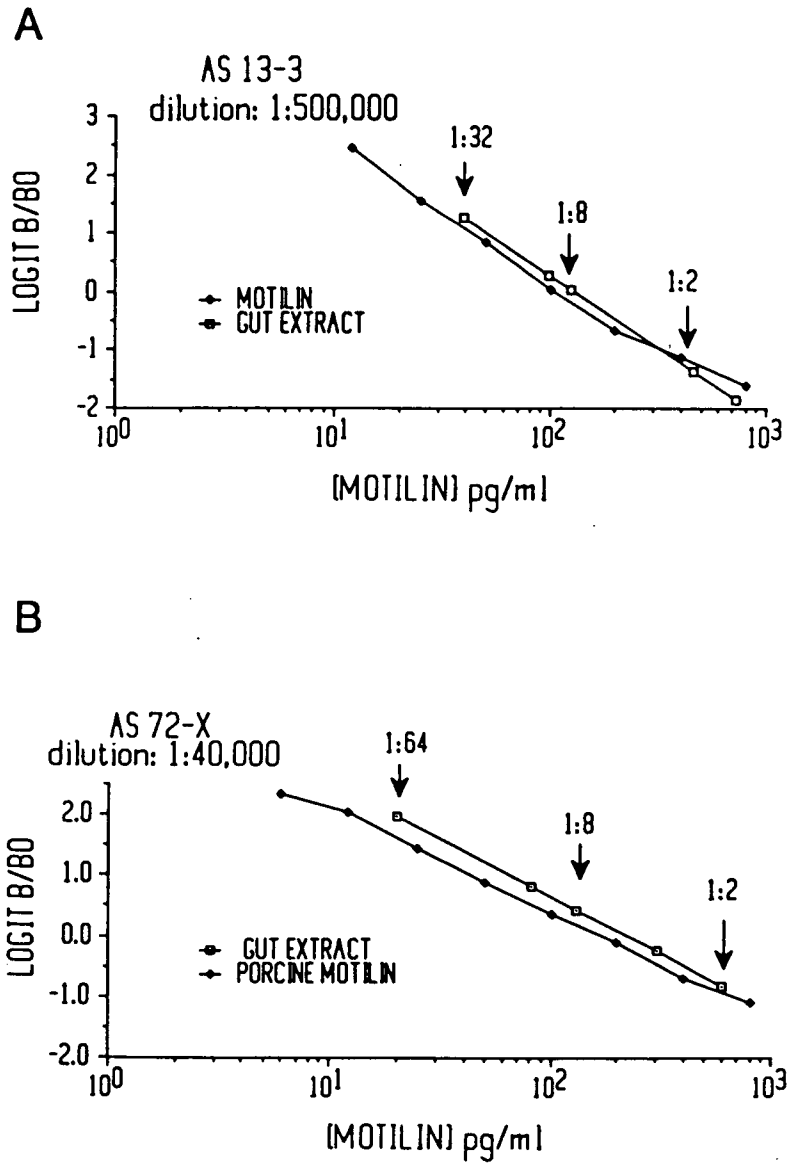


Figure 12. RIA standard curves and serial dilutions of Sep-Pak concentrated rat intestinal extracts.(A) antisera 13-3, (B) antisera 72X

				ICC RESULTS					
AS#	code	type	host	pig int./	rat int./	rat cer.	RIA	dilution	specificity
1	72x	poly	Gp	—	—	—	+	1:40k	C-term
2	13-3	poly	Rb	+	—	—	+	1:500k	C-term
3	23B	mono	My	+	—	—	n/a		?
4	M03	poly	Rb	—	—	—	—	1:10k	C/N-term
5	74	poly	Gp	—	—	—	n/a		C/N-term
6	Mx	poly	Rb	—	—	—	n/a		?

TABLE I.

The antiserum employed ; the host species in which they were produced (Gp,guinea pig. Rb, rabbit. My, mouse myeloma NS1.); their ability to react with cells in tissue from pig intestine (pig int.), rat intestine (rat int.), or rat cerebellum (rat cer.); the ability of antisera to detect IR-M in rat intestinal extracts by RIA; and the antigenic specificity of the antisera as determined by binding to C-terminal or N-terminal fragments of porcine motilin. (n/a= antiserum not applicable to RIA; no displacement of ^{125}I-motilin)

TISSUE EXTRACTION

The purification steps in the crude extraction of IR-M from 560g of rat intestinal tissue are shown in table II. For both the methanol extraction and Sep-Pak chromatography steps, yields of IR-M were approximately 70% of the previous step. In both of these steps IR-M in side fractions accounted for the remaining 30%. Yield after SP-Sephadex C-25 chromatography, however, fell to 32% of IR-M in the previous step, and the early eluting fraction from this column accounted for less than one quarter of the remaining 68%. Overall purification after SP-Sephadex C25 chromatography was approximately 19 fold.

CHROMATOGRAPHIC CHARACTERIZATION OF RAT INTESTINAL IR-M

GEL FILTRATION CHROMATOGRAPHY

Fractions #53-70 from SP-Sephadex C25 chromatography (fig 13A) were pooled and lyophilized. The lyophilized fraction was redissolved in 2ml 0.2M acetic acid and 80µl sample of this material further diluted to 0.5ml and applied to the Biogel P-10 column. The motilin like immunoreactive peak co-eluted with porcine motilin indicating a similar molecular size (fig 13B). Recovery of immunoreactive material in fractions #10-12 was approximately 90% of that applied to the column.

HPLC

The pooled IR-M peak (fractions #10-12) from gel filtration chromatography were chromatographed on the HPLC system. Elution with a linear gradient of water/acetonitrile; 0.1% TFA 15-50% over 30 min,

yielded an early peak that did not correspond to the elution time for porcine motilin (fig 14).

CONTRACTILE ACTIVITY

Fractions number 53 to 70 from SP-Sephadex C25 chromatography were pooled and lyophilized. The lyophilized material was reconstituted with 2ml dH_2O. Half of this sample was retained for further chromatographic steps and the other half utilized in contractility studies. The molar concentration of this material, if similar in molecular weight to porcine motilin, was approximately 7×10^{-5}M. Fifty microlitre aliquots were introduced to a 5 ml organ bath. Consequently the final concentration was equivalent to 7×10^{-7}M. The contractile activity induced by this material was not equivalent to that observed with a 10^{-7}M dose of porcine motilin (fig 15). The concentrated tissue extract produced a brief contractile response, and the preparation quickly returned to base line activity after a single washing. The response to 10^{-7}M motilin, however, was more prolonged. Overall muscle tension did not return to baseline even after repeated washings, and intrinsic contractile activity changed from a rhythmic to a more phasic type of response (fig 15).

Segments of rabbit duodenum demonstrated desensitization to porcine motilin. Response to a 10^{-7}M dose of motilin was significantly reduced when repeated within 4-5 min of a previous dose (fig 16). The response of the preparation to 10^{-4}M acetylcholine was used as an index of tissue sensitivity. Acetylcholine induced contraction was similar before and after motilin treatment, demonstrating that desensitization was not accompanied by a loss of overall tissue sensitivity. Pretreatment of the duodenal segment with a 10^{-7}M dose of

porcine motilin did not diminish the contractile activity of a subsequent dose of extracted IR-M (fig 17).

IMMUNOCYTOCHEMISTRY

Rigorous attempts to immunostain rat intestinal tissue for IR-M failed to produce positive results with any of the antisera employed. Two of the antisera, however, were capable of staining IR-M cells in porcine jejunum (table 1). An example of this staining and the ability to inhibit the reaction by pre-incubation* of the antiserum with porcine motilin is given in figure 18.

DISTRIBUTION OF IR-M IN THE RAT SMALL INTESTINE

Tissue from five different regions of the rat small intestine was removed and immediately frozen in liquid nitrogen. The tissue was pulverized while frozen using a mortar and pestel, then extracted for 6 h in 2% TFA. After centrifugation aliquots were lyophilized then reconstituted in assay buffer and assayed for IR-M. The two antisera capable of recognizing rat intestinal IR-M (table I), gave strikingly different distributions. Most notably antiserum 13-3 detected large amounts of IR-M in the duodenum, whereas antiserum 72X did not detect any IR-M in the duodenum (table III).

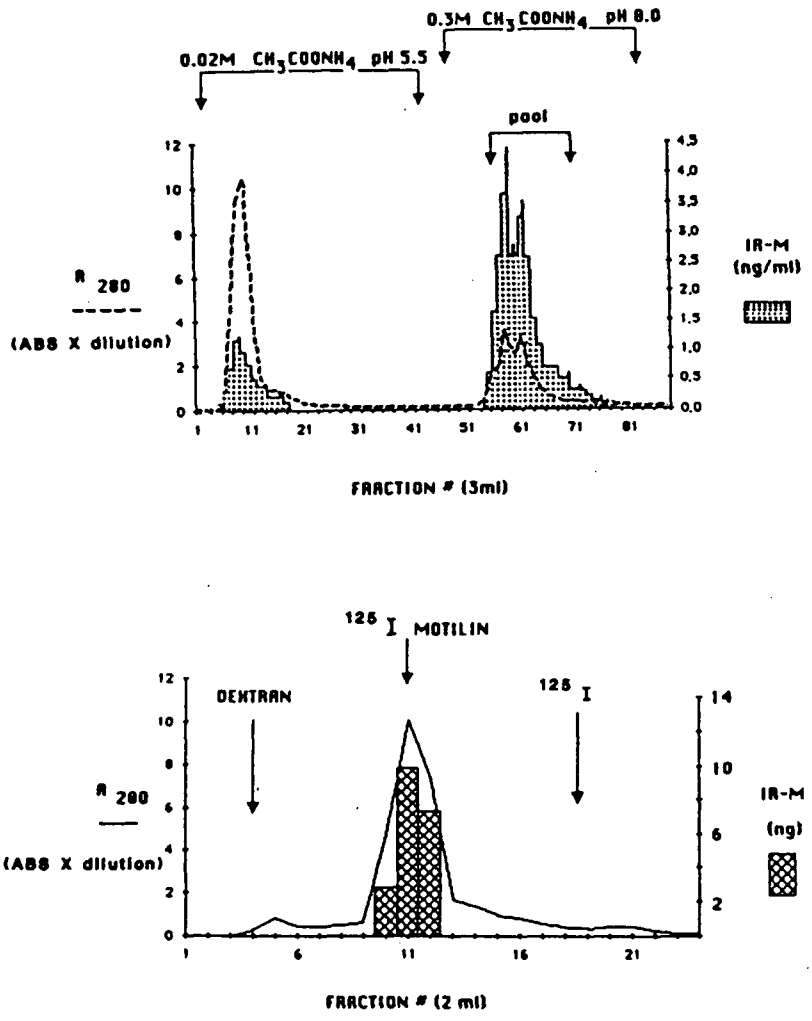


Figure 13. (A) SP-Sephadex-C25 chromatography of Sep-Pak concentrated rat intestinal extract. (B) Gel filtration (Bio-gel P-10 200-400 mesh) of pooled fractions 53-70 from SP-Sephadex-C25.

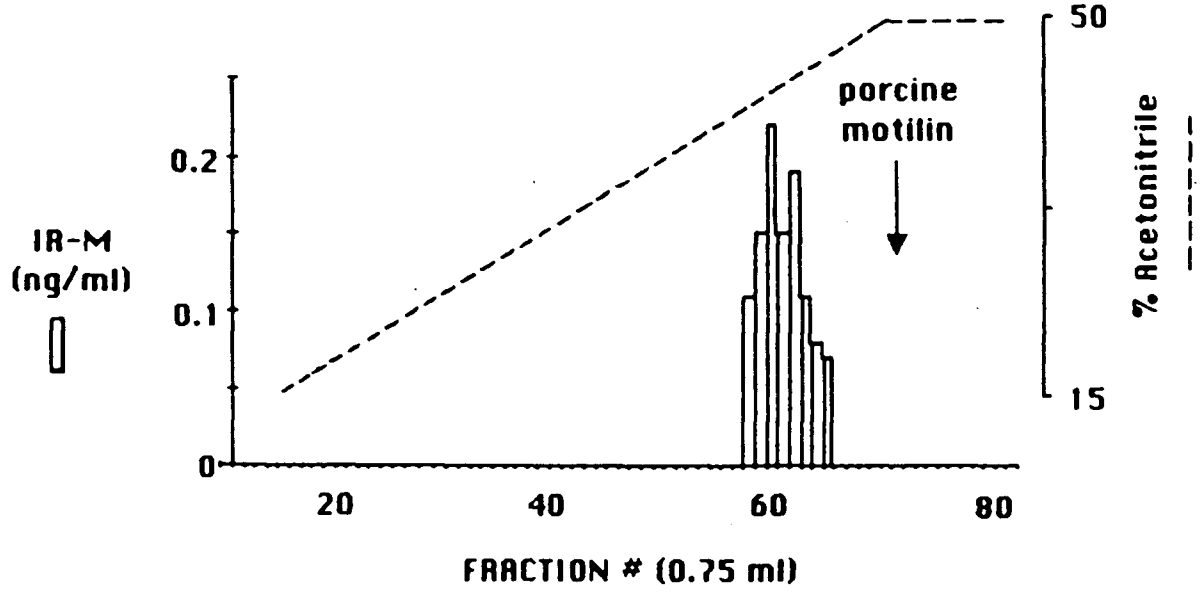


Figure 14. HPLC profile of rat intestinal extract after gel filtration.

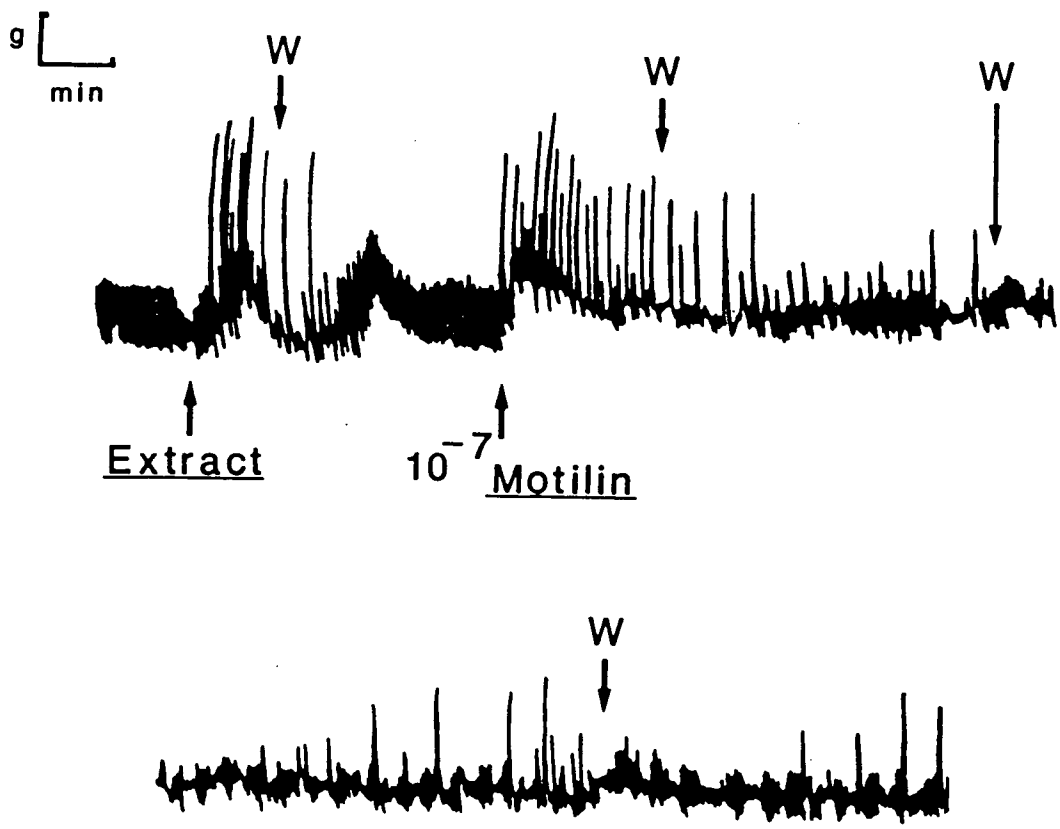
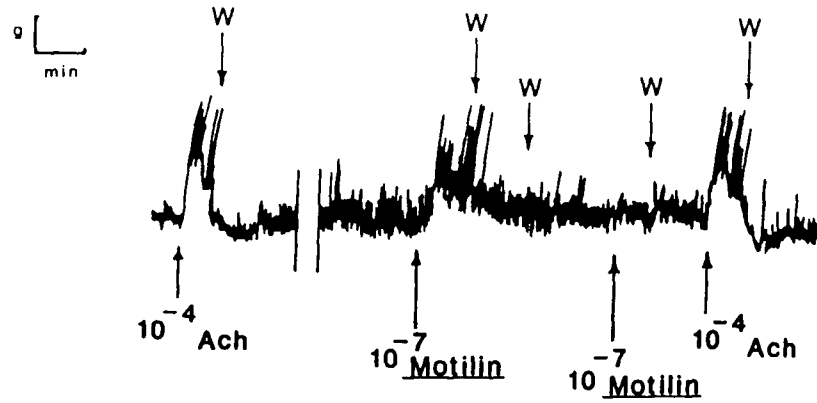


Figure 15. Contractile response of isolated rabbit duodenal muscle strip to rat intestinal tissue extract and 10^{-7}M porcine motilin. (W=wash)



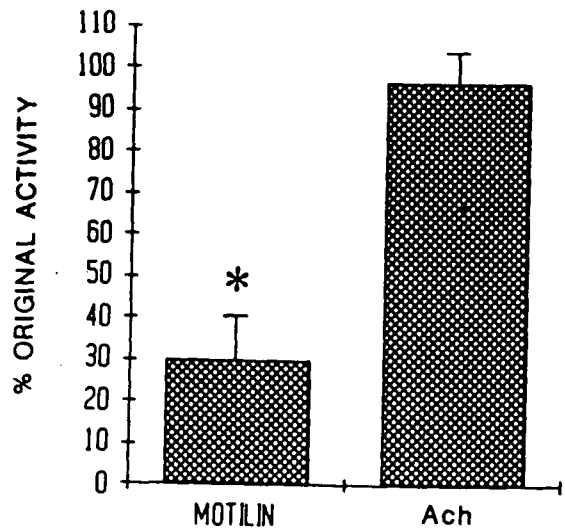
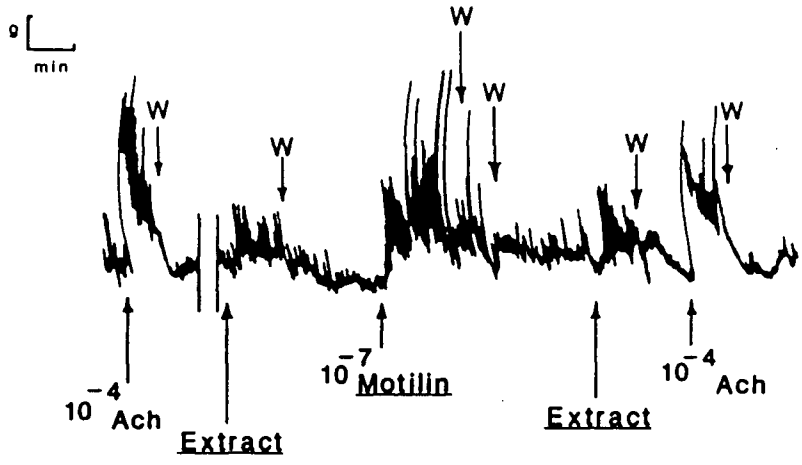


Figure 16. Motilin induced desensitization of rabbit duodenal mucscle. Histogram represents the mean + S.E.M. (N=4) contractile response as a percentage of the original response to a 10^{-7}M dose of motilin. The second response was significantly reduced compared to the response observed when the same dose was given 4 min earlier. * significant to p=0.01 (ANOVA). W = wash



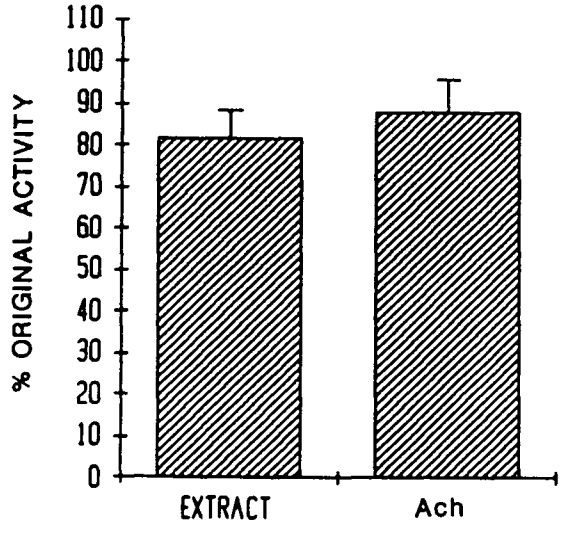
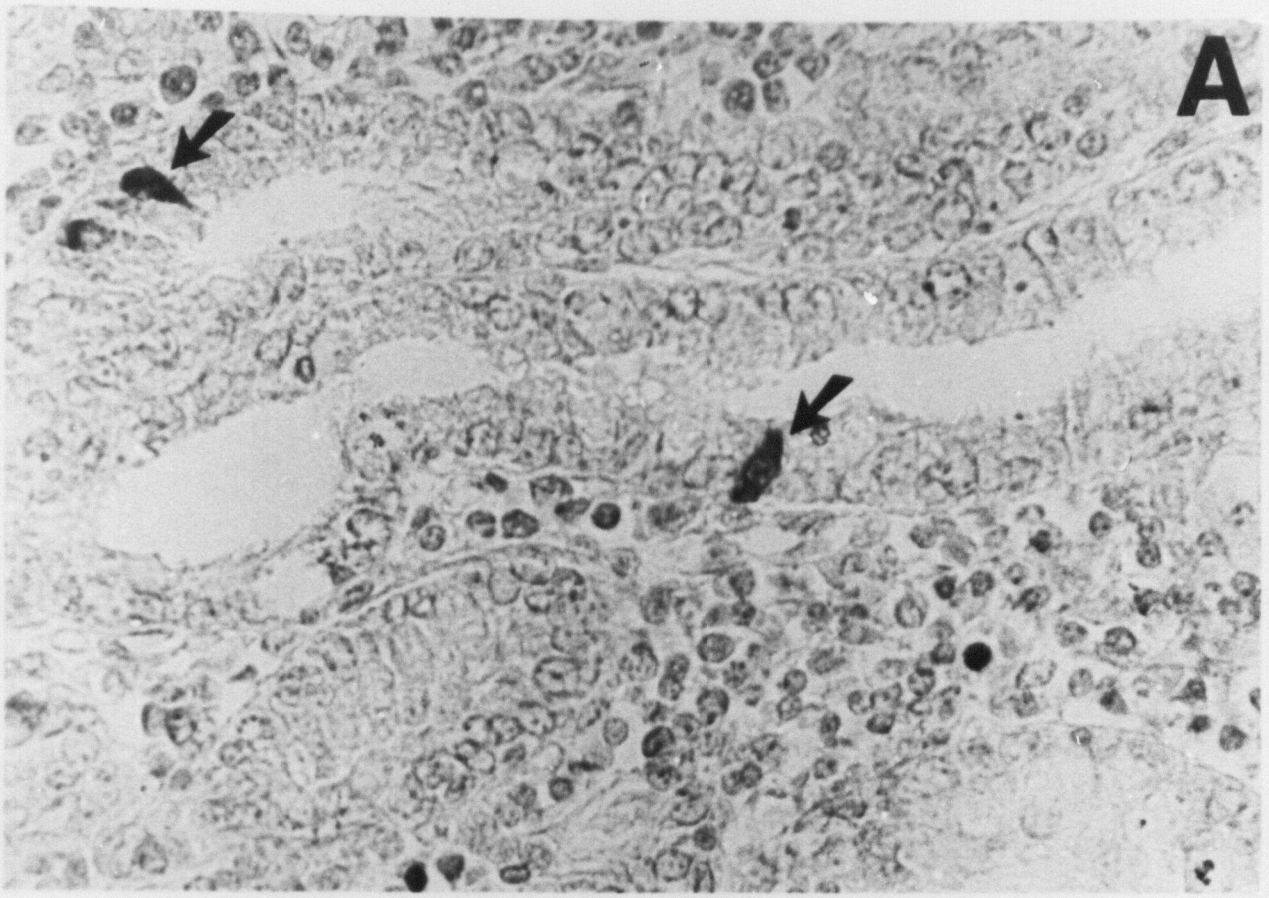


Figure 16. Motilin induced desensitization of rabbit duodenal mucscle. Histogram represents the mean + S.E.M. (N=4) contractile response as a percentage of the original response to a 10^{-7}M dose of motilin. The second response was significantly reduced compared to the response observed when the same dose was given 4 min earlier. * significant to p=0.01 (ANOVA). W = wash


A

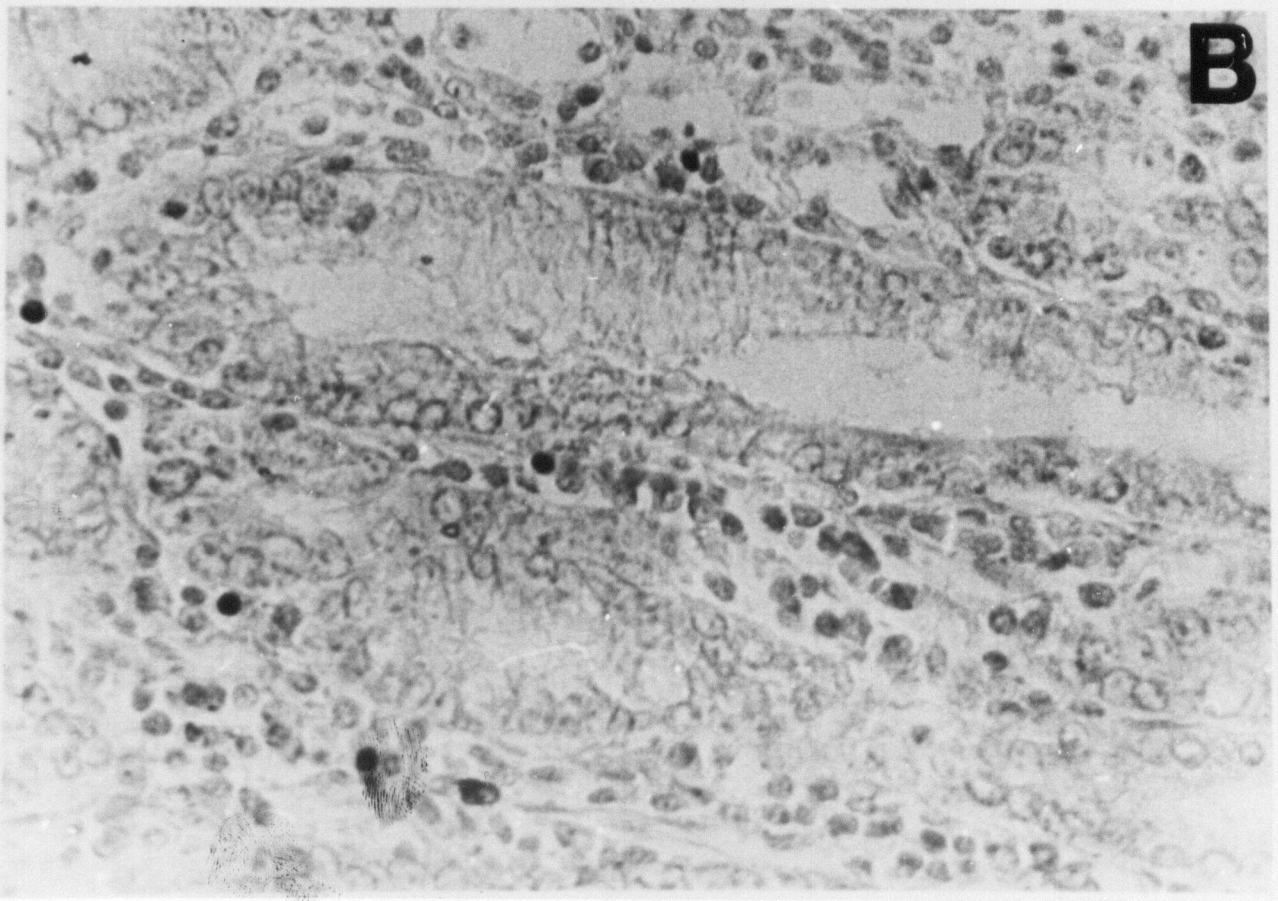

B

Figure 18. Serial sections of porcine duodenum (10 μm) . A) Arrow indicates peroxidase anti-peroxidase stained cells using motilin antiserum 13-3. B) Serial section stained identical to plate "A", but antiserum 13-3 was incubated overnight at $4^{0}C$ in lug/ml porcine motilin.

STEP	A 280 (total)	IR-M (total)	IR-M/A280	Purification	Step Purification	Yield
acid extract	14242	2800 ng	0.2	—	—	100%
methanol	2325	2085	0.9	4.3	4.3	74
Sep-Pak	790	1416	1.78	8.9	2.1	68
SP-Sephadex-C25	120	450	3.75	18.8	2.1	32

Table II.

Partial purification of rat intestinal IR-M from 560g tissue. Total absorbance: A_{280} dilution volume. Yield at each step was calculated as a percentage of the total IR-M detected in the previous step.

	Antiserum	
TISSUE	13-3	72X
corpus	1.45 ±0.21	ND (ng/g wet weight)
duodenum	4.96 ± 0.55	ND
jejunum 1	3.65 ± 0.28	1.01 ± 0.22
jejunum 2	3.62 ± 0.22	1.35 ± 0.53
ileum	3.77 ± 0.33	1.64 ± 0.21
control	0.52 ± 0.15	ND

Table III. Distribution of IR-M in the rat corpus and proximal small bowel. (Values are means ± standard error, N=4-6. jejunum 1 = proximal 30 cm, jejunum 2 = distal 30 cm. Control = abdominal muscle.)

Discussion

1) INTERDIGESTIVE INTESTINAL MYOELECTRIC ACTIVITY IN THE RAT

The interdigestive migrating myoelectric complex (MMC) has been observed in several mammalian species including the rat (17,46,48,81,96). Exogenous porcine motilin has been shown to increase the frequency of MMC in both man (97) and dog (48,101). Both species demonstrate peaks in endogenous motilin correlated with phase III of the MMC in the upper duodenum and stomach (49,50,96).

Species differences in the physiological control of the MMC appear to be associated with differences in eating habits and food sources (98). Periodic myoelectric activity seen in fasted herbivores is not disrupted by normal feeding. In omnivores such as the pig, periodic activity is not disrupted by normal feeding, but can be altered by high-calorie meals. Rat dog and man are all classified as carnivores, and in these species normal feeding abolishes the periodic myoelectrical activity associated with the fasted state.

There is an interspecies relationship between eating habits and food source, and changes in the motility pattern seen with the fed and fasted state. This relationship, however, does not necessarily imply similarities in the mechanisms for controlling these motility patterns. There are considerable similarities in the presentation of MMC's in both man and dog, including four distinguishable phases and a period of approximately 100 min (100). Exogenous motilin elicits premature activity fronts in both species (97,48). In dog, disappearance of MMC's with immunoneutralization of motilin (55,74), and the persistence of MMC's with fundic denervation (94) clearly

indicates that motilin is acting as a humoral modulator of interdigestive gastric contractile activity. Other aspects of motilin physiology, such as its effects on gastric emptying (23,27,82) and stimuli for release (23,61) are not similar in man and dog.

As is seen in both man and dog, intragastric glucose and other nutrients (81) disrupt periodic interdigestive activity in the rat. There is, however, no data available for comparison of the rat to man or dog with respect to the effects of exogenous motilin, endogenous motilin, or other peptides and neural elements on interdigestive periodic activity.

Recordings of rat interdigestive myoelectric activity in this study are comparable to previous observations although MMC periods observed, 7-10 min, were somewhat shorter than has been previously recorded; 8-12 min (72), 14-18 min (81), 11-18 min (99). Disruption of motor complexes by the administration of cysteamine is also comparable to previous reports (72), although dosage and route of administration are not similar for the two studies. Similarly, oral glucose was observed to cause disruption of motor complexes. Not previously reported, was the observation that i.v. glucose had no effect on motor complexes in the rat. This result indicates that luminal presentation of nutrients is required for changes in motility patterns. Similar results are seen in the dog where interruption of the myoelectric complex is dependent on the physical and chemical nature of the food being presented to the duodenum. When the intestine is by-passed and nutrition is given parenterally there is no disruption of periodic activity (98).

The administration of motilin and motilin antiserum had no effect on the interdigestive myoelectric activity in the rat. Similar results have been observed in the pig (18,17). The observation that porcine motilin has no effect on the MMC in rats may be due to species differences in the structure of motilin. Conversely, the lack of an effect of porcine motilin in the rat may represent a fundamental difference in the physiological control mechanisms governing interdigestive activity in the rat as compared to dog and man. Such a difference is exemplified in the pig where MMC's occur during the digestive period (18), and periodic fluctuations of plasma motilin are absent unless the animal is fasted for prolonged periods (16).

Before the structure of canine motilin was published it was suggested that all 22 amino acids of motilin were important to its biological activity. This hypothesis was based on gastric contractile activity of synthetic porcine motilin fragments in fasted conscious dogs (51). Motilin fragments 1-6 and 12-22 had no contractile activity, while fragment 7-22 demonstrated minimal activity, approximately 1/300 that of intact motilin. Replacement of the N-terminal residue of motilin also resulted in a loss of activity to less than 1/300 of the natural peptide. Surprisingly, the recent characterization of canine motilin indicates that it differs from porcine motilin at 5 of its 22 amino acids (75,80). The amino acid differences occur at positions 7, 8, 12, 13 and 14; histidine:tyrosine, serine:glycine, lysine:arginine, isoleucine:methionine, arginine:glutamine, respectively (canine:porcine). Amino acid switches at positions 8, 12 and 13

represent only minor changes in structure. The replacement of an aromatic with a basic residue at position 7 and switching of a basic for an acidic residue at position 14, however, represent significant structural alteration that might be expected to change the tertiary structure of canine motilin. Based on earlier structure activity observations (51) these differences in canine motilin would also be expected to significantly reduce the potency of this peptide. As the biological potencies of canine and porcine motilin are identical (75), it must be concluded that the pharmacophore of porcine motilin does not include the entire 22 amino acid sequence, and that this biologically active sequence is not affected by the amino acid changes seen in canine motilin.

Three generalizations can be drawn from the above discussion: 1) Man and dog, both classified as carnivores, display similar characteristics with respect to the role of motilin in the control of interdigestive gastric contractile activity. 2) The rat, also classified as a carnivore, displays characteristics indicating that control of interdigestive contractile activity may be similar to man and dog. 3) Amino acid sequence differences between canine and porcine motilin do not affect the biological activity of motilin in the dog. The rat is ostensibly within the same classification as man and dog in that it is a carnivore and interdigestive MMC's are disrupted by the luminal presentation of certain nutrients. Therefore, a natural extension of the above generalizations would be to suggest that the effects of porcine motilin in the rat should be similar to motilin induced effects in man and dog. As this does not appear to be the case

it must be concluded that the rat displays fundamental differences from other carnivores in the mechanisms controlling interdigestive activity.

ii) PORCINE-MOTILIN MONOCLONALS DO NOT DETECT IR-M IN THE RAT

Several reports have indicated that antisera directed against porcine-motilin were capable of specifically staining rat cerebellar tissue (19,68). Certain motilin antisera were also capable of detecting IR-M in extracts of rat intestine (68,54,66). Based on these observations it appeared reasonable to assume that a monoclonal antibody directed against rat intestinal IR-M could be produced using porcine motilin as the original antigen. One fusion, utilizing murine B-cells primed with porcine motilin, produced over 100 motilin positive clones none of which reacted with rat intestinal tissue. None of the serum samples collected from motilin immunized mice prior to fusion were capable of immunochemically staining rat intestinal tissue. Although ICC results were negative for mouse serum prior to fusion it was speculated that the polyclonal antiserum could possibly contain a very small fraction with the ability to bind rat IR-M, and that this small fraction although not evident in the polyclonal state, could become amplified through fusion and subsequent cloning. The failure to produce a monoclonal antibody capable of reacting with rat intestinal tissue, while inconclusive in itself, does suggest that the ability of polyclonal antisera to detect IR-M in rat intestinal extracts is artifactual in nature.

iii) CHARACTERIZATION OF RAT INTESTINAL IR-M

Motilin was originally isolated from a side fraction produced during the extraction of secretin from hog intestinal mucosa (62,63). The technique originally used for the extraction of secretin involved boiling of the tissue for 8-10 minutes. The rationale for boiling the tissue includes denaturing proteolytic enzymes as well as precipitation of the bulk of the nitrogenous mass. Following the boiling step the tissue was homogenized then acid extracted. Basic peptides were removed from the acid extract by adsorption on alginic acid. Peptides were eluted from the alginic acid and precipitated with NaCl. The ethanol soluble fraction of this concentrate was subjected to gel filtration chromatography, and the smaller molecular weight fractions extracted into methanol. The methanol soluble fractions were retained for further purification of secretin. After methanol extraction the crude secretin was subjected to ion exchange chromatography on carboxymethylcellulose (CMC). The early eluting fractions from the CMC step were the side fractions that provided the starting material for motilin extraction (13,62).

Gastrointestinal hormones are distributed diffusely throughout the gastrointestinal tract. The diffuse nature of the gastro-enteropancreatic neuroendocrine system has been the major obstacle to isolation of gastrointestinal regulatory peptides. As the pig is utilized for human consumption this species provided large quantities of starting material for the original isolation of motilin. Until recently, limited amounts of available tissue coupled with low recoveries have prevented isolation of gut peptides from species that

are not used for human consumption. Advances in purification and microsequence analysis, however, have contributed to the recent isolation and characterization of canine intestinal motilin (80). The technique utilized for the extraction of canine motilin still relies on boiling the tissue to denature proteolytic enzymes, and extraction of basic peptides with acid. Major differences in technique are seen in the methods of concentrating the various fractions. In the original isolation of porcine motilin, NaCl precipitation and lyophilization were employed, whereas Sep-Pak cartridges (Waters), and evaporation under reduced pressure were used as means of concentration in the isolation of canine motilin. Significant losses occur with salt precipitation and lyophilization as motilin is invariably trapped within precipitates in both cases. These losses are greatly reduced by keeping the motilin containing fractions in an aqueous phase, as is seen with the extraction of canine motilin (80). Another major technical advance that greatly facilitated the microisolation of canine motilin was the use of high pressure liquid chromatography (HPLC). The sensitivity and resolution of HPLC greatly exceeds that possible with classical methods of column chromatography.

In this study 560g of rat intestinal tissue was extracted. In accordance with established techniques for the extraction of gastrointestinal peptides, the tissue was first boiled for 10 min in order to denature proteolytic enzymes (62,63). After boiling, followed by extraction into 2.0% TFA, the yield was approximately 3ng/g tissue wet weight. From table III it can be seen that the average IR-M content detected in the rat small bowel by antiserum 13-3 is

approximately 4ng/g tissue wet weight. The approximately 25% reduction in IR-M detected in the bulk tissue extract as compared to the smaller samples used in the distribution study, probably reflects the trapping of immunoreactive material within the cellular debris during centrifugation. In the distribution study, tissues were extracted in proportionally larger volumes of 2.0% TFA. Although extraction into a larger volume of dilute acid appears to provide a better initial yield of immunoreactive material, the larger volumes proved to be too unwieldy in the larger extractions to justify the extra 25% recovery.

The observation that certain gastrointestinal peptides, including motilin, are soluble in low molecular weight alcohols was applied to the second stage of motilin extraction, although Mutt (62,63) applied methanol extraction to a much later step in purification of secretin (62). Extraction into methanol and subsequent precipitation with ether provided a significant concentration step as well as a four fold purification (table II). Application of methanol soluble material to Sep-Pak cartridges provided another two fold purification and further concentration. Loss of IR-M in both the methanol and Sep-Pak steps was approximately 30%. These losses could be largely accounted for in the immunoreactive content of methanol insoluble material and side fractions from Sep-Pak chromatography. With SP-Sephadex-C25 chromatography yield dropped to a prohibitive 32% of the previous step. Only 15-25% of the missing IR-M could be accounted for in the early eluting peak from SP-Sephadex-C25. The remaining immunoreactive material remained either strongly bound to the sulfonyl-propyl

residues or lost its immunoreactive properties on passage through the column. The substantial drop in yield with SP-Sephadex-C25 chromatography prevented further quantitative separation as a relatively large quantity of immunoreactive material was required for assessment of contractile activity. A small aliquot of the later eluting peak from SP-Sephadex-C25 material, however, was retained for qualitative comparisons to the elution profiles of porcine motilin on gel filtration and HPLC.

Gel filtration chromatographs of rat intestinal extracts yielded an IR-M peak that co-eluted with porcine motilin. This observation is in accordance with previously published observations (68), and indicates that rat intestinal IR-M has a molecular size similar to porcine motilin.

When pooled IR-M from gel filtration was chromatographed by reverse phase HPLC, rat intestinal IR-M did not co-elute with porcine motilin. This observation is in disagreement with previously published results (54,68). There are two possible reasons for this discrepancy. First, the extraction protocol employed in this study may have altered rat intestinal IR-M resulting in a different elution profile on reverse phase HPLC. This possibility was examined by subjecting porcine motilin to a similar extraction procedure, then chromatographing "extracted" and "unextracted" porcine motilin. The elution profile of extracted porcine motilin was identical to that of porcine motilin before extraction. A more likely reason for the discrepancy between the chromatographic properties of rat intestinal IR-M observed in this study and those of previous reports is that a

more rigorous extraction protocol is employed in this work which presumably results in a more highly purified form of IR-M being applied to the HPLC column. Chromatographic profiles of crude extracts on HPLC can be highly variable and resolution is often poor with concentrated crude samples as non-covalent bonding between peptides can form complexes that are difficult to dissociate.

A number of immunocytochemical studies have shown that motilin-containing cells of the mammalian gastrointestinal tract were located predominantly in the duodenum and jejunum (44,71). Subsequent reports have indicated a more widespread distribution and have identified motilin immunoreactivity in the mucosa of the oesophagus, stomach, ileum, and colon, as well as peripheral locations such as pancreas, gall bladder, adrenals and kidney in some mammals (33,104).

The nature of motilin containing cells has been investigated in a number of studies. Motilin immunoreactivity has been described as primarily located to a sub-population of enterochromaffin (EC) cells (77), primarily in non-EC cells (44), or located in variable proportions to both EC cells and non-EC cells (33,24). The reasons for discrepancies in motilin distribution and cell type have been variously described as 1) differences in immunoreactivity of antisera (53), 2) motilin heterogeneity (24), or 3) variation in EC cell content dependent on stages of maturation of EC cells (26).

The identification of IR-M in rat intestinal tissue is similarly plagued with discrepancies. Originally, insignificant amounts of IR-M were detected by RIA in rat intestinal extracts (88,105). Subsequently, highly variable levels of IR-M in rat intestinal tissue

have been reported (54,68). Consistent with earlier reports we have also detected relatively low levels of IR-M in rat intestinal tissue. The apparent distribution and relative amounts, however, vary significantly dependent on the antiserum used. As with discrepancies in motilin localization and distribution in other species this is presumably due to variation in antisera specificity and\or heterogeneity of IR-M.

The specificity of the antisera used in this study was only roughly gauged according to the ability of either N-terminal (1-13) or C-terminal (14-22) amino acid fragments of motilin to cross-react with the antisera. The cross-reactivity of these fragments was assessed by first incubating the antisera with the fragment and subsequently determining the resulting inhibition of binding to uncleaved motilin. Of the antisera that were assessed in this manner (antiserum MX and monoclonal 23B yielded non-specific results) there appeared to be two general groups; primarily C-terminal directed, or C-terminal directed with a large proportion also recognizing the N-terminal. Antisera 13-3 and 72X are primarily C-terminal directed. With both of these antisera binding to uncleaved porcine motilin was less than 10% inhibited when preincubated with the N-terminal fragment at a concentration of approximately 500 ng/ml for 72X and 10 ng/ml for antiserum 13-3. Maximal inhibition of binding to intact porcine motilin occurred when these antisera were pre-incubated with the C-terminal fragment at the same concentrations. Antisera M03 and 74, however, demonstrated approximately 50% and 40% inhibition of binding, respectively, when

pre-incubated with the N-terminal fragments at the same concentrations that induced maximal inhibition using the C-terminal fragment.

The observation that rat intestinal IR-M is detected only by antisera which are predominately C-terminal specific is consistent with earlier observations (88) where the highest concentrations of IR-M in rat intestine were detected using a C-terminal specific antiserum. This is suggestive of homology between the C-terminal region of porcine motilin and an unspecified region of rat intestinal IR-M. However, the rough approximation of antisera specificity to either the C-terminal or N-terminal in this study and that of Shin et. al. (88) allows for only the most tentative speculation with respect to possible homologies. For instance, both this study and that of Shin et. al. (88) demonstrated that two apparently C-terminal directed antisera could detect widely varying amounts of IR-M. Shin et. al. further characterized his C-terminal antisera into either sensitive or insensitive to amino acid substitutions at position 15. Similar characterization was not attempted in this study, but presumably similar differences in antisera 72 and 13-3 may exist and thus could explain the different distributions observed.

Rat intestinal extracts were compared to natural porcine motilin for their ability to displace ^{125}I-motilin in the motilin RIA. Representative figures are shown in figure 12A and 12B. The inhibition caused by the addition of serial dilution of rat intestinal extract is parallel to the porcine motilin standard curve. This suggests that both antisera 13-3 and 72X have similar binding characteristics for porcine motilin and rat intestinal IR-M.

The specific displacement of ^{125}I-motilin by rat intestinal IR-M was further examined by adding a standard amount of porcine motilin to rat intestinal extracts. The increased displacement of ^{125}I-motilin resulting from the addition of porcine motilin to samples of rat intestinal extract was additive and linear. These results are a further indication that rat intestinal extracts did not interfere with the binding of porcine motilin to the antisera used.

Attempts to immunostain for motilin in rat intestinal tissue have produced only negative results. There are several possible explanations for the lack of motilin-like staining in the rat intestine. It is possible that the major epitopes of rat motilin are unavailable to the antisera in the in situ preparation. This explanation seems unlikely in that antisera which are appropriate for RIA of GI peptides are in general capable of detecting those peptides by immunocytochemical techniques. Alternatively, it is possible that tissue fixation is disruptive to rat intestinal motilin. Again, this seems unlikely as a variety of fixatives were employed, and motilin-like immunoreactivity was observed in tissues from other species (pig) fixed in an identical manner. The third and most plausible explanation is that the amino acid sequence of rat intestinal motilin is different from that of porcine motilin and as a result does not cross react in situ with antiserum raised against porcine motilin. This hypothesis is supported by the observation that partially purified and concentrated rat intestinal IR-M produces contractile activity in rabbit duodenal muscle strips that is different from that induced by porcine motilin. Furthermore, there was no indication of cross-desensitization to rat

intestinal extract by pretreatment of the muscle strips with porcine motilin. This suggests that contractile activity induced by porcine motilin is not mediated by pathways similar to the contractile activity induced by rat intestinal extracts of IR-M. These observations coupled with the lack of immunocytochemical staining for IR-M in rat intestinal tissue indicate that assayable levels of IR-M in rat tissue extracts may be due to nonspecific interaction of peptides or peptide fragments with the assay. Of interest in this regard are several recent reports disputing the existence of motilin in rat brain. Motilin-like immunoreactivity in rat brain was either not detected (RIA) (34), not inhibited (ICC) by addition of porcine motilin (54,67), or did not chromatograph (HPLC) similar to porcine motilin (54). These results suggest that localization of motilin in rat brain tissue (68,4) may also result from non-specific binding of an uncharacterized fraction of the antisera. In support of this view it has been observed that several monoclonal antibodies raised against porcine motilin do not stain CNS tissue in a variety of species including the rat (S. Vincent , personal communication).

In summary, the results presented here suggest that intestinal motilin, if it exists in the rat, is present in a form that is very different from porcine motilin. The possibility that the motilin-like immunoreactive material detected in the rat intestine is artifactual has been suggested by the inability to generate monoclonal antibodies demonstrating anti-motilin activity in rat intestinal tissue. It is possible that fractions of the polyclonal antisera 13-3 and 72X react with peptide fragments generated during the extraction of intestinal

tissue. A computer search of known peptide sequences (4) has revealed that a portion of rabbit skeletal muscle tropomyosin (TM), 142-150, bears a striking similarity to the middle portion of porcine motilin (PM), 9-17.

PM	9-17.	Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-Glu
TM	142-150	Glu-Leu-Gln-Glu-Met-Gln-Leu-Lys-Glu

Although intact or heat denatured TM did not cross-react with motilin antisera, specific fragments of digested TM were not rigorously tested (4). Furthermore, only known peptide sequences have been screened for possible homology with motilin. The majority of cellular proteins have not been sequenced, and the possibility remains of other peptide fragments with homologies to one or more regions of porcine motilin. Of interest in this regard is the observation that pharmacologically active opioid peptide sequences can be derived from enzymatically treated bovine blood, and that these novel peptides originate from both cytochrome b and haemoglobin (7,8).

It also appears unlikely that a motilin-like peptide is involved in the control of interdigestive periodic activity in the rat, as exogenous porcine motilin had no effect on the MMC in the rat. In species such as man and dog where motilin does appear to act as a modulator of MMCs, the biologically active site of motilin is conserved. According to current phylogenetic theory the order Artiodactyl (pigs) and Carnivora (dogs) show divergent evolution from an early common ancestor. As motilin has been isolated and characterized in both the pig and dog, it is apparent that the

ancestral motilin-like peptide must have existed prior to divergence. The same common ancestor that gave rise to the pig and the dog also gave rise to the primates of which the order Rodentia (rats) are an early divergent lineage. Consequently, a motilin-like peptide might be expected to exist in both rats and man. As previously discussed there are reasonably good data suggesting the existence of motilin in man (plasma IR-M levels correspond to MMC cycles, and exogenous motilin induces premature MMCs). From the data presented in this study, the converse is true for the rat. The presence of motilin in widely divergent species such as pig and dog, and the high probability that motilin exists in a closely related species (man) indicates that the probable lack of motilin in the rat is due to evolutionary factors that have selected for divergence of the ancestral motilin-like gene in this species. It is apparent that motilin is relatively labile with respect to changes in its amino acid sequence; 23% difference between canine and porcine motilin. The fact that motilin is poorly conserved between species further supports the hypothesis that this peptide may have been relatively quickly selected out of certain species.

As previously mentioned, interruption of periodic activity by the luminal presentation of nutrients is characteristic of carnivores, but not of herbivores, and omnivores such as the pig will display characteristics of either group depending on its diet. In the rat, periodic activity is also disrupted by food, placing this species into the catagory including carnivores. Classifying the rat as a carnivore, however, is dubious if not misleading. The vast majority of rodents, due primarily to size restrictions, are herbivores and although the

rat may be a facultative carnivore it is likely to have evolved from a strictly herbivorous lineage. The extended periods of almost continuous food intake characteristic of herbivores, may represent a selective pressure against the expression of motilin, and/or allow mutations leading to the expression of an entirely different peptide, both functionally and immunologically.

In conclusion, two of the antisera empolyed in this study recognize an IR-M component of rat intestinal extracts. This component, however, is likely to be an artifact possibly resulting from a protein fragment that has some homology with the C-terminal portion of motilin. Thus a large scale extraction of rat intestinal tissue for motilin utilizing antisera 13-3 or 72x in the detection method, is not warranted. Furthermore, it is apparent that the rat may be significantly different from either man or dog with respect to mechanisms controlling periodic interdigestive intestinal activity. Consequently, this species may not provide an appropriate model for the study of motilin.

REFERENCES

1. Adachi H., Toda N., Hayachi S., Noguchi M., Suzuki T., Torizuka K., Yajima H., Koyama K.; Mechanism of the excitatory action of motilin on isolated rabbit intestine. Gastroenterology 80; 783-788 (1981)

2. Bayliss W.M., Starling E.H.; The movements and innervation of the small intestine. J. Physiol. 26; 125-138 (1899)

3. Bayliss W.M., Starling E.H.; The movements and innervation of the small intestine. J. Physiol. 26; 125-138 (1901)

4. Beinfeld M.C., Korchak D.M.; The regional distribution and the chemical, chromotographic, and immunologic characterization of motilin brain peptides: the evidence for a difference between brain and intestinal motilin-immunoreactive peptides. J. Neuroscience 5(9); 2502-2509 (1986)

5. Beinfeld M.C., Bailey G.J.; The distribution of motilin-like peptides in Rhesus monkey brain as determined by radioimmunoassay. Neuroscience Letters 54; 345-350 (1985)

6. Bloom S.R., Mitznegg P., Bryant M.G.; Measurement of human plasma motilin. Scand. J. Gastroent. 11 (supp. 39); 47-52 (1976)

7. Brantl V., Gramsch Ch., Lottspeich F., Henschen A, Jaeger K.H., Herz A.; Novel opioid peptides derived from mitochondrial cytochrome b: chytochrophins. Europ. J. Pharmacol. 111; 293-314, (1985)

8. Brantl V., Gramsch Ch., Lottspeich F., Mertz R., Jaeger K.H., Herz A.; Novel opioid peptides derived from hemoglobin: hemorphins. Europ. J. Pharmacol.125; 309-310, (1986)

9. Brown J C; Presence of a gastric motor stimulating property in duodenal extracts. Gastroenterology 52; 225-299 (1967)

10. Brown J C, Cook M A, Dryburgh J R; Motilin, a gastric motor activity stimulating polypeptide: The complete amino acid sequence. Can J. Biochem. 51; 533-537 (1973)

11. Brown J C, Cook M A, Dryburgh J R; Motilin a gastric motor activity stimulating polypeptide: Final purification, amino acid composition and C-terminal residues. Gastroenterology 62; 401-404 (1972)

12. Brown J C, Johnson L P, Magee D F; Effect of duodenal alkalinization on gastric motility. Gastroenterology 50; 333-339 (1966)

13. Brown J C, Mutt V, Dryburgh J R; The further purification of motilin a gastric motor activity stimulating polypeptide from the mucosa of the small intestine of hogs. Can. J. Physiol. Pharmacol. 49; 399-405 (1971)

14. Brown J C, Parkes C O; Effect on fundic pouch motor activity of stimulatory and inhibitory fractions separated from pancreozymin. Gastroenterology 53; 731-736 (1967)

15. Bormans V.,Peeters T.L., Vantrappen G.; Motilin receptors in rabbit stomach and small intestine. Reg. Peptides. 15;143-153 (1986)

16. Borody T.J., Byrnes D.J., Titchen D.A.; Motilin and migrating myoelectric complexes in the pig and the dog. J. Exp. Physiol. 69; 875-890 (1984)

17. Bueno L., Fioramonti J., Rayner V., Ruckebush Y.; Effects of motilin, somatostatin and pancreatic polypeptide on the migrating myoelectric complex in pig and dog. Gastroenterology 82; 1395-1402 (1982)

18. Bueno L., Ruckebusch Y.; Migrating myoelectrical complexes: disruption enhancement and disorganization. In: Gastrointestinal motility in health and disease. (H. Duthie, ed.) pp. 83-91 (1978)

19. Chan-Palay V.; Coexistence of traditional neurotransmitters with peptides in the mammalian brain: Serotonin and substance P in the raphe and GABA and motilin in the cerebellum. In: Co-transmission. (A.C.Cuello ed.) pp. 1-24 Macmillan. London (1982)

20. Chan-Palay V.; Immunocytochemical and autoradiographic methods to demonstrate the co-existence of neuroactive substances: cerebellar Purkinje cells have glutamic acid decarboxylase, cysteine sulfinic acid decarboxylase, and motilin immunoreactivity. Acta. Morphol. Hung. 31; 193-212 (1983)

21. Chan-Palay V., Nilaver G., Palay S.L., Beinfeld M.C., Zimmerman E.A., Wu J.Y., O' Donohue T.L; Chemical heterogeneity in cerebellar Purkinje cells: Existence and coexistence of glutamic acid decarboxylase-like and motilin-like immunoreactivities. Proc. Natl. Acad. Sci. , 78; 7887-7791 (1981)

22. Christofides N.D., Bloom S.R., Besterman H.S.; Physiology of motilin II. In: Gut Hormones (S.R. Bloom ed.) pp.343-350 Churchill Livingstone, London. (1978)

23. Christofides N.D., Bloom S.R., Besterman H.S., Adrian T.E., Ghatei M.A.; Release of motilin by oral and intravenous nutrients in man. Gut 20;102-106 (1978)

24. Christofides N.D., Bryant M.G., Ghatei M.A., Kishimoto S., Buchan A.M.J., Polak J.M., Bloom S.R.; Molecular forms of motilin in the mammalian and human gut and human plasma. Gastroenterology 80;292-300 (1981)

25. Cook M.A., Kowalewski K., Daniel E.E.; The use of the isolated, homologous perfused canine stomach in the study of the loci and mechanism of action of gastrointestinal hormones. Materia Medica Polona 9; 113-117 (1977)

26. Dawson J., Bryant M. G., Cox A.G., Christofides N.D., Bloom S.R., Peters T.J.; Subcellular localization of serotonin and motilin in the human jejunum. Clin. Sci. 59; 505-508 (1980)

27. Debas H.T., Yamagishi T., Dryburgh J.R.; Motilin enhances gastric emptying of liquids in dogs. Gastroenterology 73; 777-780 (1977)

28. Enriques E., Hallion L.; Reflexe acid de Pavloff et secretine: mechanisme humoral commun. C.R. Soc. Biol. Paris 55; 233-234 (1903)

29. Forssman W.G., Yanaihara N., Helmstaedtler V., Grube D.; Differential demonstration of the motilin-cell and the enterochromaffin cell. Scand. J. Gastroent., [Supp. 11] 39; 43-45 (1976)

30. Fox J.E.T.; Motilin - An update. Life Sciences. 35;695 (1984)

31. Fox J.E.T., Daniel E.E., Jury J., Fox A.E., Collins S.M; Sites and mechanisms of action of neuropeptides on canine gastric motility differ in vivo and in vitro. Life Sciences 33; 817-825 (1983)

32. Fox J.E.T., Daniel E.E., Jury J., Robotham H.; The mechanism of motilin excitation of the canine small intestine. Life Sciences 34; 1001-1006 (1984)

33. Fox J.E.T., Track N,S., Daniel E.E., Yanaihara N.; Immunoreactive motilin is not exclusive to the gastrointestinal tract. Biomed. Res. 2; 321-324 (1981)

34. Fratta W., Panula P., Yang H-YT., Costa E.; Biochemical and immunohistochemical evidence for the presence of motilin in pig cerebellum. Brain Res. 341;171-175 (1985)

35. Furness J.B., Costa M.; Projections of intestinal neurons showing immunoreactivity for vasoactive intestinal polypeptide are consistent with these neurons being the inhibitory neurons. Neuroscience Lett. 15; 199-204 (1979)

36. Furness J.B., Costa M.; Types of nerves in the enteric nervous system. Neuroscience 5; 1-20 (1980)

37. Goodfriend T.L., Levine L., Fasman G.D.; Antibodies to bradykinin and angiotensin: A use for carbodiimide in immunology. Science 144; 1344-1346 (1964)

38. Grey W.R.; Sequential degradation plus dansylation. In: Methods in enzymology. 11; (C.H.W. Hirs, ed.) pp. 469 Acedemic Press, New York. (1967)

39. Grider J.R., Cable M.B., Said S.I., Maklouf G.M.;Vasoactive intestinal peptide as a neural mediator of gastric relaxation. Am J. Physiol. 248; G73-G78 (1985)

40. Gross E., Witkop B.; Selective cleavage of methionyl peptide bonds in ribonuclease with cyanogen bromide. J. Am. Chem. Soc. 83; 1510-1519 (1961)

41. Gross E., Witkop B.; Nonenzymatic cleavage of peptide bonds: The methionine residue of bovine pancreatic ribonuclease. J. Biol. Chem. 237; 1856-1860 (1962)

42. Hall K.E., Greenburg G.R., El-Sharkawy T.Y. Damian W.E.; Vagal control of migrating motor complex related peaks in canine plasma motilin, pancreatic polypeptide and gastrin. Can. J. Physiol. Pharm. 61; 1289-1298 (1983)

43. Heitz P U, Polak J M, Kasper M, Timson, C M, Pearse A G E; Immunoelectron cytochemical localization of motilin and substance P in rabbit bile duct enterochromaffin (EC) cells. Histochemistry 50; 319-325 (1978)

44. Helmstaedtler V., Kreppein W., Domschke W., Mitznegg P., Yanaihara N., Wunsch E., Forsmann W.G.; Immunohistochemical localization of motilin in endocrine nonenterochromaffin cells of the small intestine of humans and monkeys. Gastroenterology 76; 897-902 (1979)

45. Hirning L.D., Thomas F.B.; Neurogenic mechanism of action of motilin in the canine isolated small intestine ex vivo. Europ. J. Pharm. 128; 241-248 (1986)

46. Holloway R.M., Blank E., Takahashi I., Dodds W.J., Layman R.D.; Motilin: A mechanism incorporating the opossum lower esophageal sphincter into the migrating motor complex. Gastroenterology 98; 507-515 (1985)

47 Hunter W.M., Greenwood F.C.; Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature (London) 194; 495 (1962)

48. Itoh Z., Aizawa I., Takeuchi S., Couch E.F.; Hunger contraction and motilin. In: Proceedings of the fifth international symposium on Gastrointestinal motility. (G. Vantrappen ed.) pp.48-55. Typoff, Belgium. (1975)

49. Itoh Z., Sekiguchi T.; Interdigestive motor activity in health and disease. Scand. J. Gastroent. 18,(supp. 82): 121-134 (1983)

50. Itoh Z., Takeuchi S., Aizawa I., Takayanagi R., Mori K., Taminato T., Seino Y., Imura H., Yanaihara N.; Changes in plasma motilin concentration and gastrointestinal contractile activity in conscious dogs. Dig. Dis. 23; 929-935 (1978)

51. Itoh Z., Takeuchi S., Aizawa I., Takayanagi R., Mori K., Taminato T., Seino Y., Imura H., Yanahaira N.; Recent advances in motilin research: Its physiological and clinical signficance. In: Gastrointestinal hormones and pathology of the digestive system. (M. Grossman, V. Speransa, N. Basso, E. Lezoche, ed's) pp. 241-258 Plenum Press New York (1977).

52. Keast J.R., Furness J.B., Costa M.; Somatostatin in human enteric nerves.:Distribution and characterization. Cell Tiss. Res. 237;299-308 (1984)

53. Kishimoto S., Polak J.M., Buchan A.M.J., Verhofstad A.A., Steinbusch J.W., Yanaihara N., Bloom S.R., Pearse A.G.; Motilin cells investigated by the use of region-specific antisera. Virchows Arch. 36;207-218 (1981)

54. Lange W., Unger H.P., Wencl A.; Is motilin a cerebellar peptide in the rat? A radioimmunological, chromatographic and immunohistochemical study. Anat. Embryol 173;371-376 (1986)

55. Lee K.Y., Chang T.M., Chey W.Y.; Effect of rabbit antimotilin serum on myoelectric activity and plasma motilin concentration in fasting dog. Am. J. Physiol. 245; G547-G553 (1983)

56. Lemoyne M., Wassef R., Tasse D., Trudel L., Poitras P.; Motilin and the vagus in dogs. Can. J. Physiol. 62; 1042-1096 (1984)

57. Maklouf G.M.; Enteric neuropeptides: Role in neuromuscular activity of the gut. Trends in Pharmacological Sciences 6 ; 214-218 (1985)

58. McIntosh C.R.S., Brown J.C., Motilin. In: Advances in metabolic disorders 11; (R. Levine, R. Luft, ed's) Acedemic Press. New York (in press)

59. Mishell B.B., Shiigi S.M.; Selected methods in cellular immunology. pp.361, Feeman and CO., San Francisco (1980)

60. Modlin I.M., Mitznegg P., Bloom S.R.; Motilin release in the pig. Gut 19; 399-402 (1978)

61. Mori K., Seino Y., Itoh Z., Yanaihara N., Imura H.; Motilin release by intravenous infusion of nutrients and somatostatin in conscious dogs. Reg. Peptides 1; 265-270 (1981)

62. Mutt V.; Preparation of highly purified secretin. Arkiv for Kemi. 15(8); 69-74 (1959)

63. Mutt V.; Hormone Isolation In: Gut Hormones. (S.R. Bloom, ed.) pp. 21-27 . Churchill Livingstone. (1978)

64. Mutt V.; Chemistry of the gastrointestinal hormones and hormone-like peptides and a sketch of their physiology, and pharmocology. Vitamines and Hormones 39; 231-427 (1982)

65. Nakazawa S., Segaw K., Hidano H., Tsukamoto Y., Aichi M., Ichikawa T.; The effect of motilin on gastrointestinal propulsion in rats. Gastroenterology Jap. 13; 50-52 (1978)

66. Nilaver B., Defandi R., Zimmerman E.A., Beinfeld M.C., O' Donohue T.L.; Motilin related immunoreactivity in purkinge cells of the mammalian cerebellum. Soc. Neurosci. Abstr. 7; 99 (1981)

67. Nilaver G., Defandi R., Zimmerman A., Beinfeld M.C, O'Donhue T.L.; Motilin in the Purkinje cell of the cerebellum. Nature 295; 597-598 (1982)

68. O'Donohue T.L., Beinfeld M.C., Chey W.Y., Chang T., Nilaver G., Zimmerman E.A., Yajima H., Adachi H., Poth M., Mcdevitt T., Jacobowitz D.M; ; Identification, characterization, and distribution of motilin immunoreactivity in the rat central nervous system. Peptides 2;476-477 (1981)

69. Ormsbee H.S., Telford G.L., Mason G.R.; Required neural involvement in control of canine migrating motor complex. Am.J. Physiol. 237; E451- E460 (1979)

70. Peeters T.L., Vantrappen G., Janssens J.; Fasting plasma motilin levels are related to the interdigestive motility complex. Gastroenterology 79; 716-719 (1980)

71. Pearse A.G.E., Polak J.M., Bloom S.R., Adams C., Dryburgh J.R., Brown J.C.; Enterochromaffin cells of the mammalian small intestine as the source of motilin. Virchows Arch. [Cell Path.] 16; 111-120 (1974)

72 Pihan G., Kline T.J., Hollenberg N.K., Szabo S.; Duodenal ulcerogens cysteamine and proprionitrile induce gastroduodenal motility alterations in the rat. Gastroenterology 88; 989-97 (1985)

73. Pilot M.A., Thomas P.A., Grill R.E., Knight M.A., Ritchie D.H.;Hormonal control of the gastric motor response to feeding: cross-perfusion of an isolated stomach using a conscious donor dog. World J. Surg. 6; 422-426 (1982)

74. Poitras P.; Motilin is a digestive hormone in the dog. Gastroenterology 87; 909-913 (1984)

75. Poitras P, Reeve J R, Hunkapiller M W, Hood L E, Walsh J H; Purification and characterization of canine intestinal motilin. Reg. Pep. 5; 197-208 (1983)

76. Poitras P., Steinbach J.H., VanDeventer G., Code C.F., Walsh J.H.; Motilin independent ectopic fronts of the interdigestive myoelectric complex in dogs. Am. J. Physiol. 239; G215-220 (1980)

77. Polak J.M., Heitz P., Pearse A.G.; Differential localization of substance P and motilin. Scand. J. Gastroent. 11;39-42 (1976)

78. Polak J.M., Pearse A.G.E., Heath C.M.; Complete identification of endocrine cells in the gastrointestinal tract using semithin-thin section to identify motilin cells in human and animal intestine. Gut 16;225-229 (1975)

79. Popielski L.; Uber die physiologische wirkung von extrakten aus samtlichen teilen des verdauungshanales (Magen, Deck-und Dundarm) sowie des gehirns pankreas und blutes und uber die chemischen eigenschafen des darin wirkenden korpers. Pflugers Arch. Physiol. 128;191-221 (1909)

80. Reeve J R, Ho F J, Walsh J H, Ben-Avram C M, Shively J E; Rapid high-yield purification of canine intestinal motilin and its complete sequence determination. J . Chromatog. 321; 421-432 (1985)

81. Ruckebush M., Fiorimonti J., Fioramonti S. ; Electrical spiking activity and propulsion in small intestine in fed and fasted rats. Gastroenterology 68: 1500-1508 (1975)

82. Ruppin H., Domschke S., Domschke W., Wunsch E., Jaeger E., Demling L.; Effects of 13-Nle-motilin in man - Inhibition of gastric evacuation and stimulation of pepsin secretion. Scand. J. Gastroent. 10;199-202 (1975)

83. Sarna S., Stoddard C., Belbeck L., Mcwade D.; Intrinsic nervous control of migrating myoelectric complexes. Am. J. Physiol. 241; G16-G23 (1981)

84. Sarr M.G., Kelly K.A., Go V.L.W.; Motilin regulation of canine interdigestive intestinal motility. Dig. Dis. Sci. 28; 249-256 (1983)

85. Segawa T.,Nakano M., Kai Y., Kawatani H., Yajima H.; Effect of synthetic motilin and related polypeptides on contraction of gastrointestinal smooth muscle. J. Pharm. Pharmacol. 28;650-651 (1976)

86. Shay H, Gershon-Cohen J; Experimental studies in gastric physiology in man. II A study of pyloric control . The roles of acid and alkali. Surg. Gynec. Obstet. 58, 939-955 (1934)

87. Shay H, Gershon-Cohen J; Experimental studies in gastric physiology in man: The mechanisms of gastric exacuation after partial gastrectomy as demonstrated roentgenologically. Am. J. Dig. Dis Nut. 2; 608-613 (1935)

88. Shin S., Imagawa K., Shimizu F., Hashimura E, Nagai K., Yanaihara C., Yanaihara N.; Heterogeneity of immunoreactive motilin. Endocrinol. Jap. 27(supp. 1) ;141-149 (1980)

88b. Shubert H., Brown J.C.; Correction to the amino acid sequence of porcine motilin. Can. J. Bioch. 52; 7-8 (1974)

89. Solcia E., Capella C., Buffa R., Usillini L., Fiocca R., Frigerio B., Tenti P., Sessa F.; The diffuse endocrine-paracrine system of the gut in health and disease: Ultrastructural features. In : Structure of the gut. (M.H. Polak, S.R. Bloom, N.A. Wright, M.J. Daly, ed's) pp. 25-36 Glaxo Group Research LTD. , U.K. (1982)

90. Solcia E., Polak J.M., Larsson L.I., Buchan A.M.J., Cappela C.; Update on Lausanne classification of endocrine cells.In: Gut Hormones. second ed. (S.R. Bloom, J.M. Polak, ed's) pp. 96-100 Churchill Livingston , Edinburgh (1981)

91. Strunz U., Domschke W., Mitznegg P., Domschke S., Shubert E., Wunsch E., Jaeger E., Demling L.; Analysis of the motor effects of 13-Nle-motilin on the rabbit ,guinea pig, rat and human alimentary tract in vitro. Gastroenterology 68;1485-1491 (1975)

92. Szurszewski J.H.; A migrating electrical complex of the canine small intestine. Am. J. Physiol. 217; 1757-1763 (1969)

93. Thomas J.E., Crider J O, Morgan J C; A study of reflexes involving the pyloric sphincter and antrum and their role in gastric evacuation. Am. J. Physiol. 198; 683-700 (1934)

94. Thomas, P.A., Kelly K.A., Go V.L.W.; Does motilin regulate canine interdigestive gastric motility? Dig. Dis. 24; 577-582 (1979)

95. Vantrappen G.; In: Motility of the digestive tract.(M., Wienbeck ed.) pp. 157-167. Raven Press, New York (1982)

96. Vantrappen G., Agg H.O., Janssens J., Peeters T.L.; The migrating motor complex. Medical Clin. North Am. 65(6); 1311-1329 (1981)

97. Vantrappen G., Janssens J., Peeters T.L., Bloom S.R., Christofides N., Hellmans J.; Motilin and the interdigestive migrating motor complex in man. Am. J. Dig Dis. 24; 497-500 (1979)

98. Weisbrodt N.W., Copeland E.M., Thor P. J.; The myoelectrical activity of the small intestine of the dog during total parenteral nutrition. Proc.Soc. Exp. Biol. Med. 153; 121-124 (1976)

99. Weisbrodt N.W., Sussman S.E., Stewart J.J., Burks T.F.; Effect of morphine sulfate on intestinal transit and myoelectric activity of the small intestine of the rat. J.Pharm Exp. Therap. 214 (2); 333-338 (1980)

100. Wingate D. L.; The small intestine. In: A guide to gastrointestinal motility (J. Christensen, D.L Wingate., ed's). pp. 128-156. Wright PSG, Boston (1983)

101. Wingate P.L.; Backwards and forwards with the migrating complex. Dig. Dis. Sci 26; 641-666 (1981)

102. Woods K.R., Wang K.T.; Separation of dansyl-amino acids by polyamide layer chromatography.Biochim. Biophys. Acta. 133; 369-370 (1967)

103. Wunsch E., Brown J.C., Deimer K.H., Drees F., Jaiger E., Musiol J., Scharf R., Stocker H., Thamm P., Wendlgerger G.; The total synthesis of norleucine-13-motilin. Zeit. Naturforsch 28; 234-240 (1973)

104. Yanaihara C., Sato H., Yanahaira N., Haruse S., Forssman W.G., Helmstaedter V., Fujuta T., Yamaguchi K., Abe K.; Motilin, substance P, and somatostatin-like immunoreactivities in extracts from dog, tupia and monkey brain and GI tract. Adv. Exp,. Med. Biol. 106; 269-283 (1977)

105. Yanaihara N., Yanaihara C., Nagai K., Sato HG., Shimizu F., Yamaguchi K., Abe K.; Motilin-like immunoreactivity in porcine, canine, human and rat tissues. Biomed. Res. 1; 76-83 (1980)